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THE BACTERIOPHAGE TYPING OF STAPHYLOCOCCI

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Sensitivity to bacteriophage has been used as an aid in the classification and differentiation of individual strains of several bacterial species, and has been applied in recent years to studies on the staphylococci. In 1935 Burnet and Lush presented evidence to show that certain strains of *Staphylococcus aureus* could be grouped according to their sensitivity to a series of phages.[1] Subsequently, Williams and Timmins used 4 phages isolated by Burnet and Lush to distinguish 6 types among strains of staphylococci which had been obtained in cultures from patients with osteomyelitis.[2] In their work phage action was indicated by the clearing of broth cultures of the staphylococci.

In 1942 Fisk described the isolation and propagation of phages from lysogenic cultures of *Staph. aureus*.[3] The phages were propagated by serial passage on agar cultures of specific susceptible strains of staphylococci, and were carried to the point where the undiluted phage suspensions produced confluent lysis of the cultures. These phages were derived from coagulase-positive strains and none produced lysis of coagulase-negative staphylococci. Sensitivity to the phages was indicated by the development of areas of lysis, ranging from confluent lysis to isolated plaques in cultures on agar plates upon which a small drop of each phage had been "spotted" at spaced intervals. In 2 series of tests Fisk submitted 95 and 78 cultures, respectively, to the action of 26 and 33 phages.[3,4] The strains were separated into a number of groups, each group being characterized by its susceptibility to a different combination of phages. Significantly, when staphylococci were isolated from sources which could be expected to yield identical strains, e.g., from a bone lesion and from the blood in a case of osteomyelitis, they exhibited sensitivity to the same phages, while the dissimilarity of unrelated strains was shown by their susceptibility to different groups of phages.

Using Fisk's methods, Fisher submitted 140 strains of coagulase-positive staphylococci to a series of 16 phages which she had isolated from lysogenic cultures.[5] Of these, 103 (73.5%) were assigned to 30 different groups on the basis of their susceptibility to various combinations of phages. The remaining 37 strains were not lysed by any of the 16 phages.

In 1945 Wilson and Atkinson[6] modified Fisk's method to permit the differentiation of staphylococci in a manner somewhat comparable to that which had

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1. Burnet, F. M. and Lush, D. 1935, J. Path. & Bact. **40**: 455–469.
2. Williams, S. and Timmins, C. 1938, M. J. Austral. **2**: 687–689.
3. Fisk, R. T. 1942, J. Infect. Dis. **71**: 153–160; 161–163.
4. Fisk, R. T. and Mordvin, O. E. 1944, Am. J. Hyg. **40**: 232–238.
5. Fisher, M. L. 1945, Dissertation, The Ohio State University.
6. Wilson, G. S. and Atkinson, J. D. 1945, Lancet, **1**: 647–648.

been used successfully by Craigie and his associates in phage typing of the typhoid bacilli.[7] The important modification introduced by Wilson and Atkinson involved the use of a "test dilution" of each staphylococcal phage; this was defined as the highest dilution of the phage which produced confluent lysis of its propagating strain on agar.

Using a series of 18 phages of lysogenic origin, Wilson and Atkinson found that some strains of staphylococci characteristically were lysed by certain groups of phages while others were susceptible only to single phages. With confluent lysis as the criterion for differentiation, they established 21 "phage types" and subtypes. Strains were assigned to their types 1, 2 and 3 on the basis of lysis by 3 distinct groups of phages; within each of these groups the phages acted in various combinations, 3 subtypes of types 1 and 3 and 4 subtypes of type 2 being identified by the particular combinations which produced lysis. Strains in the remaining types, 4 to 14 inclusive, were reported to be lysed only by single phages. Of 460 strains which were submitted to the 18 phages, Wilson and Atkinson assigned 278 (60.4%) to phage types or subtypes; 104 (22.6%) strains were susceptible to some phages but gave reactions which did not permit their being placed in types, and 78 (17%) were not lysed by any phage of the series. Subsequent to this report a number of additional phages have been isolated. These phages and their propagating strains of staphylococci are now maintained by the National Collection of Type Cultures, Medical Research Council, in London.

Phage typing has been employed successfully in a number of studies on the staphylococci and staphylococcal infections, particularly in Britain and Australia. Judging from the relative scarcity of reports in the American literature, it appears that little use of the method has been made in similar investigations in this country. We believe that phage typing of staphylococci promises to be of value in certain kinds of laboratory and clinical study. It is our purpose to present in this preliminary report a description of the methods of phage typing and observations on its use in the study of strains of staphylococci which were isolated from related sources. In subsequent articles it is planned to report additional data, including the application of phage typing to a study of antibiotic-resistant staphylococci.

7. Craigie, J. and Brandon, K. F. 1936, J. Path. & Bact. **43**: 233–248; Craigie, J. and Felix, A. 1947, Lancet, **1**: 823–827; Craigie, J. and Yen, C. H. 1938, Canad. Pub. Health J. **29**: 448–463.

METHODS

The methods used in this laboratory for the isolation, propagation and titration of staphylococcal phage and for phage typing are described. It should be pointed out that the details can be modified to a certain extent without affecting the results essentially.

Culture mediums.—A good nutrient medium should be employed, capable of supporting active growth of the staphylococci so as to provide a suitable substrate for phage action. Tryptose agar and beef-infusion agar, containing 0.1 to 0.2% of dextrose, were used by Fisk and by Wilson and Atkinson, respectively, and have been employed by others. In this laboratory solid mediums prepared by adding 2% of agar to tryptose-phosphate broth or brain-heart-infusion broth have been used with generally satisfactory results. However, an occasional lot of tryptose-phosphate agar appeared to inhibit the action of phage and a more suitable medium was sought. This was found in trypticase-soy broth and agar, obtained in dehydrated form from the Baltimore Biological Laboratories, and these 2 mediums are now being used exclusively in work with staphylococcal phages.

Agar plates are poured the day before they are to be used and are held overnight at 37 C, for it is essential that the surface of the medium be dry. Stock cultures of staphylococci are main-

tained on agar slants at room temperature in screw-capped tubes. Before use, transfers are made to fresh slants and then to broth.

Isolation of phage.—The detection and isolation of phage from lysogenic cultures of staphylococci is based on Fisk's method.[2] In principle this involves growing 2 strains of staphylococci together as a mixed culture on agar; should one strain carry a latent phage to which the other is susceptible, its presence is indicated by the appearance of plaques in the culture. The phage can then be isolated and propagated, with enhancement of its activity, by a number of serial passages on agar cultures of the susceptible strain.

Parallel streaks about 1 by 6 cm in extent are made on the surface of an agar plate from 18-hour broth cultures of several strains of *Staph. aureus*. When the seeded areas have dried, 1 loopful of each broth culture is spotted at spaced intervals on the streaks as illustrated in the following diagram:

		"Spots" Strain numbers			
		1	2	3	4
"Streaks"	Strain no. 1	—0—	—0—	—0—	—0—
	Strain no. 2	—0—	—0—	—0—	—0—
	Strain no. 3	—0—	—0—	—0—	—0—
	Strain no. 4	—0—	—0—	—0—	—0—

Alternatively, the entire surface of the plate may be inoculated with one strain, upon which substrate each of several other broth cultures are spotted at spaced intervals; reciprocal tests with 20 to 25 cultures can be made in this manner, each strain serving in turn as the substrate upon which the other cultures are spotted. When the phage inoculums are dry, usually a matter of 5 minutes or less, the plate is inverted and incubated for 4 to 6 hours at 37 C and held at room temperature for an additional 18 hours. The presence of phage is shown by the development of lysis at the site of a spotted culture, usually in the form of isolated plaques and less often as semiconfluent or confluent lysis (fig. 1A). As the phage may be carried by either of the cultures involved, the susceptible and carrier strains must now be identified. The material in the lysed area is rubbed up with a loop, suspended in about 0.2 ml of broth, and centrifuged. A loopful of the supernatant is spotted on freshly seeded areas of each strain on agar. The culture which now is lysed represents the susceptible strain which will be used to propagate the phage; the carrier, or lysogenic, culture is not lysed by the phage suspension (fig. 1B). The phage may be purified by plating out the phage suspension with the propagating strain and fishing an isolated plaque to about 0.5 ml of broth; 2 or 3 serial platings are adequate. Since phages of lysogenic origin often are highly selective in their action, it frequently is necessary to perform a large number of cross tests of this nature when one is searching for latent phage in a series of cultures of unknown lysogenic capacity.

Propagation of phage.—Stock preparations of freshly isolated phages and new batches of stock phages are prepared as follows. With a swab the surface of an agar plate is seeded from an 18-hour broth culture of the propagating strain. When dry, several drops of the phage suspension are spread over the plate, leaving a small margin to serve as a control of the bacterial growth. The inoculum is allowed to dry, the plate is inverted and incubated at 37 C for 4 to 6 hours, and then is placed in the refrigerator at 4 C for an additional 18 hours. Usually on the first passage of a newly isolated phage the reaction is one of isolated plaques or, at best, of semiconfluent lysis (fig. 1C). The lysed area is washed off with 1 ml of broth and the resulting suspension of phage and some cocci is centrifuged. A few drops of the supernatant are used to inoculate a freshly seeded plate of the propagating strain, the plate is incubated as described above, and the resulting area of lysis again is washed off in broth and centrifuged. Serial passages are continued in this manner until confluent lysis with little or no secondary growth occurs (fig. 1D). This usually is attained after 8 or 10 passages of newly isolated phages; with stock phages fewer passages are required. At this point it is advisable to do a preliminary titration of the phage suspension to determine whether a satisfactory potency has been reached or whether further passages are necessary.

When a satisfactory titer is reached, stock phage is prepared in a volume sufficient for typing. In this laboratory a volume of about 15 to 20 ml usually is adequate; larger quantities are not always desirable because of the tendency of some phages to lose potency during storage. Six agar plates of the propagating strain are prepared and inoculated with the phage suspension, diluted 5-fold if necessary, which was obtained from the last of the serial passages. After incubation at 37 C and at 4 C (fig. 1E), each plate is washed off with 2.5 ml of broth; the washings are pooled and centrifuged at high speed. The supernatant may be filtered for long-term storage. In our experience a considerable amount of phage sometimes is lost by adsorption on Seitz or Selas filters, and for day-to-day tests we often use the clear supernatants which are stored at 4 C with

no added preservative.

The stability of the phages varies from one strain to another. Certain phages remain potent for well over a year when stored at 4 C, while others lose potency within a few months and must be rebuilt at frequent intervals. A solution to this problem would appear to be to lyophilize the phage preparations.

Titration of phage.—From an 18-hour broth culture of the propagating strain 2 or 3 parallel streaks, each about 1 by 6 cm in extent, are made on an agar plate. A series of dilutions of the phage in broth is prepared, ranging from 1:10 to 1:10,000 for a phage of unknown potency or including a number of dilutions around the known probable potency of a stock phage. With a loop 2 mm in diameter, 1 loopful of each phage dilution is spotted at spaced intervals on the streaks. The plate is inverted and incubated at 37 C for 4 to 6 hours and is then held at room temperature for an additional 18 hours.

When the dilutions are suitably chosen, the lower dilutions produce circular areas of completely confluent lysis within which some slight secondary bacterial growth occasionally is observed. The highest dilution which produces confluent lysis represents the "test dilution" which is to be used in typing. Dilutions higher than this produce either semiconfluent lysis or isolated plaques. Figure 1F illustrates the titration of a phage with a series of dilutions designed to provide a preliminary estimation of its potency. The dilutions in this test range from 1:10 (top row, left) to 1:10,000 (bottom row, right). An arrow indicates the test dilution, 1:1000, which produced confluent lysis with slight secondary growth. In the same row, the dilution to the right is 1:3000, which gave only semiconfluent lysis. In this example, the next step would be to titrate with a series of dilutions covering a narrow range between 1:1000 and 1:3000 to determine the test dilution more exactly.

The test dilution of each phage is characteristic for that phage and remains fairly constant, with minor variations above or below that titer, as each successive new lot of phage is prepared. The test dilutions of the phages used in this laboratory range from 1:100 to about 1:5000, with the majority ranging between 1:100 and 1:1000. Stock phages are retitrated every few weeks to make certain that the preparations are of suitable potency. It is advisable frequently to set up reciprocal tests with all stock phages in their test dilutions and the several propagating strains of staphylococci as a control of their selectivity of action.

Typing of staphylococci.—One agar plate is used for each strain to be typed. With a swab the surface of the plate is seeded uniformly from an 18-hour broth culture and the inoculum is allowed to dry. Then a small amount of the test dilution of each phage is deposited on the plate at spaced intervals about 1 cm apart, using either a loop 2 mm in diameter or a small pipette drawn out to a very fine capillary. Only freshly prepared dilutions or dilutions not over 48 hours old are used. After the phages have dried the plate is inverted and incubated for 4 to 6 hours at 37 C and at room temperature for an additional 18 hours. It is convenient to rule the bottom of the plate into 1-cm squares with a glass-marking pencil, each phage dilution being placed in the center of a square. As many as 36 phages thus can be spotted on a single plate. The reading of results is aided and a direct comparison of the reactions of the cultures is facilitated if the phages are always placed on the plates in an established order.

The reactions are recorded according to the degree of lysis as follows: confluent lysis with or without secondary growth, +++; semiconfluent lysis, ++; isolated plaques, +; no lysis, −. Strains are differentiated on the basis of their sensitivity to one or more members of several distinct groups of phages, as will be described below. While some cultures are lysed by only a single phage, the majority are sensitive to several phages, acting in a variety of combinations to produce so-called patterns of lysis on the plates. In any given lytic pattern variation from confluent lysis to isolated plaques may occur. For the differentiation of strains, greatest weight is given to confluent or semiconfluent lysis; the occurrence of isolated plaques appears to indicate minor variations in sensitivity, but lysis only in this degree occasionally provides the only evidence upon which a strain can be classified.

RESULTS

In the work being reported, 25 phages were employed. Twenty-one of these were isolated originally by Wilson and Atkinson and were received through the courtesy of Dr. R. T. Fisk. To these were added 4 phages which were isolated in this laboratory from lysogenic cultures of *Staph. aureus*.

At present the authors recognize 6 broad subdivisions of coagulase-positive staphylococci, based on the reactions given by 290 strains with distinct groups

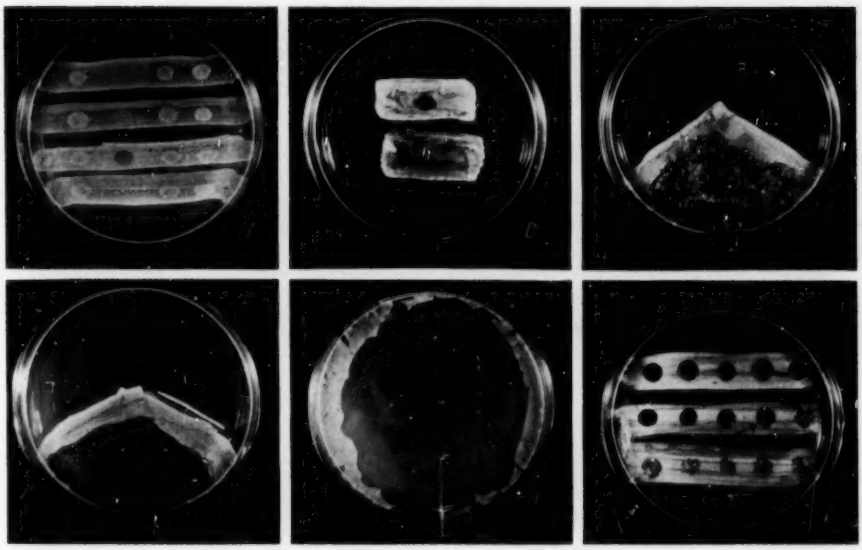

FIG. 1.—Isolation and propagation of staphylococcal bacteriophage.
A, detection of latent phage.
B, identification of carrier and sensitive strains.
C and D, stages in the propagation of phage.
E, whole plate for use in preparation of stock phage.
F, titration of phage to determine the test dilution.

of phages. While the term is not the most appropriate for broad grouping, we have retained "phage type" as a convenient designation for these subdivisions until a more definitive schema of identification can be devised. The phage types and the groups of phages* which are responsible for their identification are:

Type 1—phages 3A, 3B, 3C, 51, 39 and 523.
Type 2—phages 6, 7, 42B, 42C, 42D, 42E, 47, 47A, 47B, 47C and VA4
Type 3—phages 29, 29A, 31, 52 and 52A
Type 4—intermediate between types 2 and 3
Type 5—phages 44 and 44A
Type 6—phage 142

Types 1, 2 and 3 are comparable to the types 1, 2 and 3 established by Wilson and Atkinson. Among the phages responsible for the identification of each are the same basic phages which Wilson and Atkinson used originally to differentiate their three types. Beyond this, the schema of Wilson and Atkinson has been modified in two respects: subtypes have been omitted, and strains which are lysed by only a single phage have been assigned to one of the broad phage types. A small group of strains is distinguished by susceptibility to several of the phages responsible for the identification of both types 2 and 3; these strains appear to represent a border-line

* Phages which are isolated from lysogenic cultures of staphylococci are identified by number. To distinguish those strains which are derived by adaptation, a letter is added to the serial number of the parent phage. Phages 39, 142, 523 and VA4 were isolated in this laboratory.

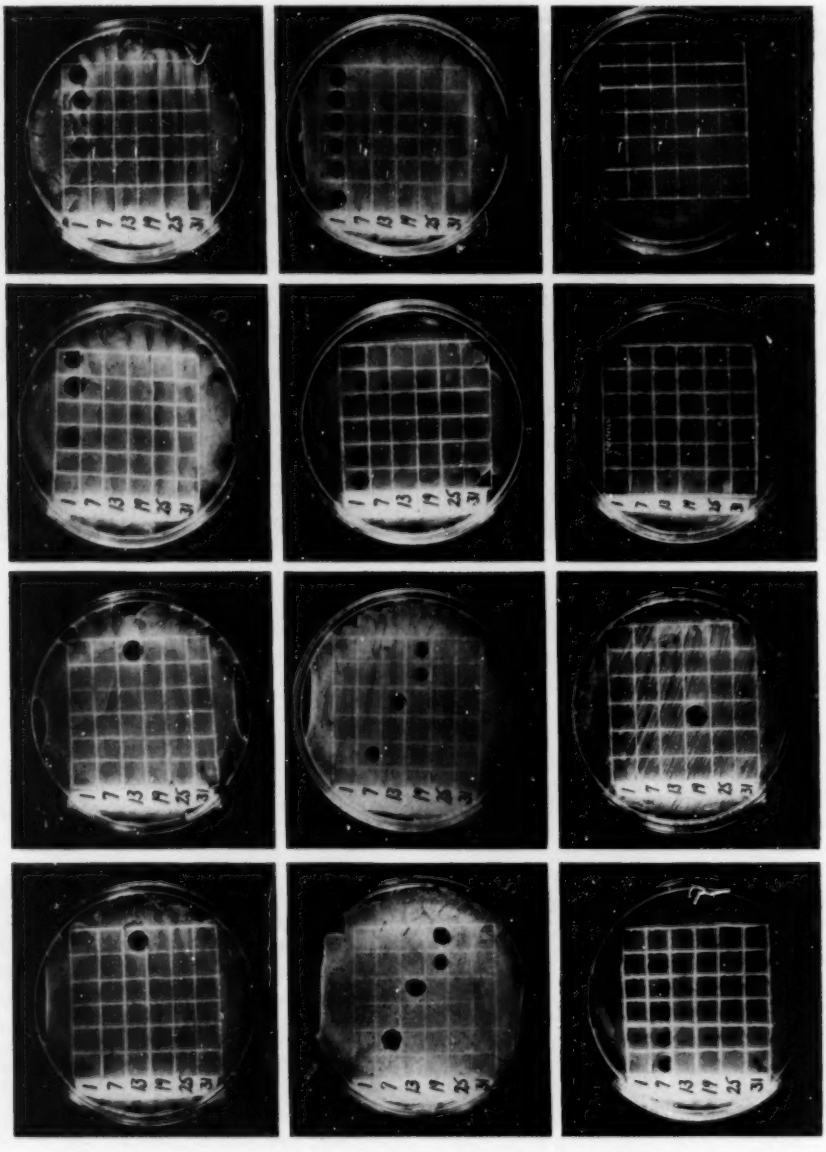

FIG. 2.—Bacteriophage typing of 6 pairs of related strains of staphylococci.
1. Case 1. Blood culture; carbunculosis.
2. Case 1. Carbuncle; 1 day later.
3. Case 2. Blood culture; acute osteomyelitis of R. ilium.
4. Case 2. R. ilium; at operation 2 days later.

type intermediate between types 2 and 3, and they have been assigned tentatively to a new type, 4. In type 5 have been placed strains which are lysed by phages 44 and 44A or by either phage alone. Rare strains are lysed only by phage 142 and by no other phage of the series; these have been assigned to type 6.

Because of the complex combinations of phages which are possible within the broad phage types, notably in types 1, 2 and 3, it does not seem advisable to establish rigid subtypes. Instead, we prefer to report the sensitivity of strains by using the identifying numbers of the phages involved. This is in line with current practice as it is encountered in the British literature and employed in the Staphylococcal Reference Laboratory of the Medical Research Council.[8] Thus, instead of referring to phage types or subtypes by number, as was the case in the schema of Wilson and Atkinson a culture which is lysed by phages 6 and 47 is reported to be of the 6/47 phage type or pattern. or a culture which is lysed only by phage 52A is regarded as belonging to phage type 52A. This procedure appears to have merit, for it records the exact spectrum of lytic reactions of a given strain and also, because of the particular phage or phages involved, it indicates the broad subdivision to which the strain is related.

Except for the reactions which suggested the establishment of type 4, we have encountered relatively few instances of cross reaction between strains belonging to one phage type and the phages responsible for the identification of the other types. These few cross reactions usually were represented by weak reactions of little apparent significance given by phages 44 and 44A with occasional strains of the first three types.

Some strains of staphylococci are not lysed by the test dilutions of the phages. Certain of these strains can be typed by resorting to the use of a series of undiluted phages. However, there is some chance that cross reactions may occur when this is done and the results must be interpreted with caution.

A total of 539 strains of coagulase-positive staphylococci was submitted to phage typing with 25 phages. Of these, 290 (53.6%) were assigned to phage types, 4 (0.7%) gave equivocal results and could not be assigned to type, and 245 (44.7%) were not lysed by any phage of the series.† In comparison, it will be recalled that Wilson and Atkin-

8. Williams, R. E. O. 1950, Descriptive literature on phage typing of staphylococci, issued by the Staphylococcal Reference Laboratory, Public Health Laboratory Service, London.

† Recently 9 additional phages were received through the courtesy of Dr. S. T. Cowan, Curator of the National Collection of Type Cultures, Medical Research Council, London, and at the present writing 34 phages are being used for typing. About 100 strains which were not sensitive to the original 25 phages were retested with the 9 new phages without materially affecting the statistical results.

←

5. Case 10. Chronic osteomyelitis of L. tibia; at operation.
6. Case 10. Operative wound, L. tibia; 23 days later.
7. Case 14. Blood culture; acute osteomyelitis of R. tibia.
8. Case 14. R. tibia; at operation 15 months later.
9. Case 24. Tuberculous abscess of L. thigh.
10. Case 24. Operative wound after excision of tuberculous sinus of L. thigh; 6 years later.
11. Case 26. Draining sinus; chronic osteomyelitis of L. humerus.
12. Case 26. Abscess of L. upper arm; 2 years later.

Numbers were placed at the left margin of the plates as a guide in "spotting" the phages and to facilitate the comparison of results. They bear no relation to the serial numbers of the phages.

son were able to type 60.4% of 460 strains of *Staph. aureus*, using 18 of the same phages employed in the present work, while Fisher typed 73.5% of 103 strains with a series of phages which she isolated. The typable strains were distributed among the 6 phage types in approximately the following proportions: type 1, 24%; type 2, 26%; type 3, 38%; type 4, 4%; type 5, 7%; and type 6, 0.8%.

Identification and differentiation of strains from related sources.—A significant application of phage typing is its use in the comparison of strains of staphylococci which have been isolated from related sources. Fisk reported that cultures obtained from sources which might be expected to yield identical strains often exhibit identical patterns of lysis when submitted to his series of phages.[3,4] Thus, the staphylococci isolated in repeated blood cultures from a given patient showed identical susceptibility to the phages, while in bacteremia associated with conditions such as cellulitis, osteomyelitis, meningitis or soft tissue abscesses the patterns of lysis of the cocci from the blood and from the lesion often were the same. Comparable observations were made by Fisher[5] and by McClure and Miller,[9] and similar results were obtained in the work now being reported.

In table 1 are recorded the results of phage typing of 70 strains of staphylococci, representing cultures which were obtained on 2 or more occasions from each of 30 patients. Cases 1 through 19 demonstrate instances in which strains showing identical phage sensitivities were isolated at intervals of from 1 day to 11 years from the same or different sources in 19 individuals. Photographs 1 to 8 in figure 2 illustrate the phage typing of 4 pairs of strains in this series. In brief, it may be pointed out that in a number of acute infections when cultures were made at intervals of a few days identical strains of staphylococci were shown to be present both in the blood stream and in a soft tissue infection or a lesion of the bone. In addition, from chronic infections in which material could be obtained for culture after intervals of several months, or even years, staphylococci sometimes were isolated which exhibited phage susceptibilities identical with that of the original infecting strain. In these latter cases the results of phage typing indicate that it is possible for the original strain of staphylococcus to persist in the body for long periods of time. Striking evidence of the persistence of the original infecting strain is seen in cases 13 and 14. Both represent instances of acute osteomyelitis of a tibia which yielded *Staph. aureus* in blood culture. In both, the acute toxic phase of the infection was treated conservatively by the administration of staphylococcal antitoxin and necessary supportive measures, but without any operative intervention.[10] Both patients responded well to this therapy and the local lesions subsided. Fifteen to 18 months later both patients suffered an exacerbation of the infection, and at this time an operation at the site of the original lesion was required. Cultures taken of the bone then yielded *Staph. aureus* of the same phage type as that of the strain which had been obtained earlier from the blood. In case 13, intermediate between the original infection and an exacerbation at the original site, osteomyelitis of the fibula occurred; an operation was performed immediately and a strain of *Staph. aureus* was isolated from

9. McClure, W. B. and Miller, A. M. 1946, Canad. M. A. J. **55**: 36–39.

10. Kleiger, B. and Blair, J. E. 1943, Arch. Surg. **46**: 548–554.

the fibula which proved to be of the same phage type as the strains obtained originally from the blood and subsequently from the tibia.

Cases 20 through 30 in table 1 illustrate instances in which strains of different phage types were isolated at intervals of from a few days to 6 years from the same or different sources in 11 individuals. In several cases the cultures were obtained from persistent, draining sinuses or from open wounds, lesions which present an obvious opportunity for contamination from exogenous sources. In such lesions it is not unlikely that the bacterial flora might change from time to time and that different strains of staphylococci might be encountered in repeated cultures; that this sometimes occurs is illustrated by cases 21, 22 and 24. Nor is it surprising that cultures of a draining sinus and from a related deep-seated infection sometimes yield strains of different phage types, as shown by cases 20 and 23. In case 28 a strain of a given phage type was isolated from the blood and an untypable strain was obtained in a culture of pus aspirated from the ankle 24 hours later. This was a fulminating, fatal infection, and we attribute etiologic significance only to the strain isolated in blood culture; the second strain, although coagulase-positive, appears to represent fortuitous contamination, probably from the skin. Cases 29 and 30 represent instances of osteomyelitis yielding *Staph. aureus* of a given phage type in cultures of the bone taken at operation, while untypable strains were obtained 4 years later when a second operation at the same site was performed. In both cases the second operations were required because of an exacerbation of the infection, and in one the probability of exogenous contamination was indicated by the presence of a draining sinus extending to the bone. The phage typing of 2 pairs of strains in this series are shown in figure 2, photographs 9 to 12.

A number of cultures which were retyped at intervals over a period of about 3 years exhibited reasonably consistent patterns of lysis. While variations in the degrees of lysis sometimes were observed, qualitative differences were not common and, when they occurred, were usually of a minor nature. This, and the fact that strains of identical sensitivity often can be isolated in repeated cultures from one individual after rather long intervals, lend added significance to those instances in which repeated cultures from the same or closely related sources yield strains of different phage types.

Epidemiology of staphylococcal infections.—Phage typing has been used to good advantage in tracing the origin and spread of certain staphylococcal infections. Wilson and Atkinson described 2 outbreaks of pemphigus in maternity homes in which phage typing provided evidence that the infections originated with members of the personnel who were staphylococcal carriers.[6] Hobbs reported the occurrence of several cases of breast abscess in which staphylococci of the same phage type were isolated from the lesions and from the throat of a nurse who was a persistent carrier of staphylococci.[11] Fisher likewise described hospital infections in which the spread was traced by means of phage typing.[5] In studies on infections due to antibiotic-resistant staphylococci in hospital populations, Barber and Rountree, respectively, and their associates have demonstrated a relationship between the strains isolated from the lesions of patients and those which were found in cultures from carriers among the attend-

11. Hobbs, B. C. 1944, Month. Bull., Min. Health, Emerg. Pub. Health Serv., Med. Res Counc. **3**: 11.

TABLE 1.—*Phage typing of coagulase-positive staphylococci isolated from related sources.*

Case no.	Strain no.	Source	Interval between first and subsequent cultures	Phage type
1	464	Blood culture; carbunculosis.		44A
	464a	Carbuncle.	1 day	44A
2	465	Blood culture; acute osteomyelitis of R. ilium.		3C, 39, 523
	465a	R. ilium; at operation.	2 days	3C, 39, 523
3	516	Blood culture; acute osteomyelitis of R. tibia.		39
	516a	R. tibia; at operation.	2 days	39
4	931	Blood culture; acute osteomyelitis of R. ulna.		3C
	932	R. ulna; at operation.	4 days	3C
5	807	Periarticular abscesses of R. hip; aspiration.		3C
	812	Periarticular abscesses of R. hip; incision and drainage.	4 days	3C
	940	Periarticular abscesses of R. hip; aspiration.	9 months	3C
6	3-6	Draining sinus; chronic osteomyelitis of L. tibia.		52A
	3-13	L. tibia; at operation.	7 days	52A
7	942	Purulent discharge following spine fusion.		42B, 42E, 47, 47B, 44A
	943	Same source.	7 days	42B, 42E, 47, 47B, 44A
	950	Same source.	24 days	42B, 42E, 47, 47B, 44A
	953	Same source.	5 weeks	42B, 42E, 47, 47B, 44A
8	424	Blood culture; generalized staphylococcal infection.		52A
	424a	Abscess of R. thigh.	16 days	52A
9	476	Blood culture; exacerbation of chronic osteomyelitis of L. femur.		3A, 3B, 3C, 51, 39, 523
	476a	L. femur; at operation.	23 days	3A, 3B, 3C, 51, 39, 523
10	805	Chronic osteomyelitis of L. tibia; at operation.		7, 42C, 42D, 42E
	817	Operative wound.	23 days	7, 42C, 42D, 42E
11	811	Draining sinus; chronic osteomyelitis of R. femur; before operation.		523
	831	Persistent sinus at site of operative wound; R. femur.	57 days	523
12	887	Purulent discharge following open reduction of fracture; L. tibia.		7, 44A
	955	Discharging sinus; same site.	6 months	7, 44A
13	523	Blood culture; acute osteomyelitis of L. tibia.		3A, 3B, 51
	523a	Osteomyelitis of R. fibula; first operation at this site.	13 months	3A, 3B, 51
	523b	Exacerbation of osteomyelitis of L. tibia; first operation at this site.	18 months	3A, 3B, 51
14	515	Blood culture; acute osteomyelitis of R. tibia.		3A, 3B, 3C, 51, 39, 523
	515a	Chronic osteomyelitis of R. tibia; first operation at this site.	15 months	3A, 3B, 3C, 51, 39, 523
15	437	Blood culture; pyarthrosis of R. knee.		42B
	437a	Pyarthrosis of R. knee; aspiration.	15 months	42B
16	762	Chronic osteomyelitis of L. hip; at operation.		52A
	762a	Chronic osteomyelitis of L. hip; at operation.	16 months	52A
17	432	Axillary abscess.		51
	432a	Folliculitis.	19 months	51
18	308	Abscess of R. shoulder; aspiration.		29
	308a	Chronic osteomyelitis of pelvis; at operation.	6 years, 8 months	29
19	478	Acute osteomyelitis of R. tibia; aspiration.		3B, 3C, 39
	478a	R. tibia; at operation.	5 days	3B, 3C, 39
	851	Chronic osteomyelitis of R. tibia; at operation.	11 years	3B, 3C, 39
20	12-6	Draining sinus; chronic osteomyelitis of L. tibia.		NT
	12-9	L. tibia; at operation.	3 days	52, 52A
	12-17	Operative wound; first post-operative dressing.	11 days	52, 52A
21	861	Discharging sinus following bone graft.		NT
	864	Same source.	12 days	52A
	945	Same source.	6 months	44A
22	809	Purulent discharge following amputation.		523, 44A
	809a	Same source.	2 days	NT
23	870	Draining sinus; chronic osteomyelitis of R. femur; at operation.		NT
	876	R. femur; at operation.	3 days	3B, 3C, 51, 523

TABLE 1.—*Phage typing of coagulase-positive staphylococci isolated from related sources (continued).*

Case no.	Strain no.	Source	Interval between first and subsequent cultures	Phage type
24	719	Tuberculous abscess; L. thigh.		6, 7, 42B, 47, 47C
	719a	Same source.	14 days	76, 77
	719b	Tuberculous sinus; L. thigh.	2 years	NT
	719c	Same source.	3 years	53, 54, 75, 77
	862	Operative wound after excision of tuberculous sinus tract.	6 years	52A
25	880	Draining sinus; lumbar region.		44A
	896	Catheterized urine; nephritis.	28 days	52A
26	704	Draining sinus; chronic osteomyelitis of L. humerus.		44A
	704a	Abscess; L. upper arm.	23 months	3A, 3B, 3C
27	740	Ulcer; L. lower leg.		44A
	740a	Abscess; L. ankle.	3 years	53, 54
28	411	Blood culture; generalized staphylococcal infection.		44A
	412	Abscess; R. ankle.	1 day	NT
29	727	Chronic osteomyelitis of L. femur; at operation.		44A
	727a	Same site; at operation.	4 years	NT
30	754	Chronic osteomyelitis of L. humerus; at operation.		44A
	754a	Same site; at operation.	4 years	NT

NT—strain not lysed by any phage of the series.
Three strains (cases 24 and 27) were lysed by some of the phages recently received from Dr. Cowan.

ing personnel.[12] In outbreaks of staphylococcal food poisoning the incrimination of specific foods has been demonstrated by showing that staphylococci isolated from the food were identical in phage type with strains obtained in cultures of the vomitus or feces of patients.[9,13,14] The source of the staphylococci which contaminated the food has been traced by the demonstration that strains isolated from the food in question, from the vomitus of the persons affected and from the nasopharynx or skin of the food handlers were of identical phage type.[14,15]

In the course of the present work we were privileged to examine a number of strains, all resistant to penicillin, which were isolated during a series of staphylococcal infections in babies at the Bronx Hospital in New York City. These have been described in detail by Felsen and his associates[16] and the cultures were sent to us by Dr. A. J. Weil. The strains were isolated from superficial pustular lesions, abscesses, empyema, blood and environmental sources such as bassinets, baby oil and the nursery floor. Of 21 strains which were submitted to phage typing, 13 (62%) were lysed by phage 52A; these included strains from various lesions, from a bassinet and a sample of baby oil. Two strains fell into other phage types and the remainder were not sensitive to the phages. In additional studies at the Bronx Hospital

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with phages supplied by this laboratory, 63% of the strains isolated from the nasopharynx or skin of babies and attending nurses and from environmental sources in the nursery were found to belong to phage type 52A.[16] Significantly, among 54 strains of *Staph. aureus* isolated from various infections in other wards of the hospital, only 5 (9%) belonged to phage type 52A, a fact which indicated that an endemic focus of infection by this type existed in the nursery and that the majority of infections in the nursery were caused by strains of a common identity.

Relation of phage type to serologic type.—It is well known that the specificity of action of many phages closely parallels the antigenic structure of the bacterial cells involved, although the factors which determine antigenicity and adsorption of phage on the cell probably are not the same. Preliminary tests were run to determine whether this holds true for the staphylococci. Strains which had been received from Dr. S. T. Cowan[17] as representative of his 3 serologic types of *Staph. aureus* were submitted to phage typing. Each of the serologic type strains tested was found to be sensitive to different phages representative of 3 distinct phage types, as follows: serologic type I, phage type 3; serologic type II, phage type 1; and serologic type III, phage type 2. Further investigation of these relationships is planned.

DISCUSSION

The phage typing of staphylococci is limited at the present time by certain factors which require considerable further study. With the phages now available it is possible to type not more than about 60% of strains of *Staph. aureus*. This immediately suggests that search for additional staphylococcal phages is necessary, either by the adaptation of some of the present phages to new cultures or by the isolation of new strains of phage. Certain strains of staphylococci exhibit a lowered sensitivity to phage, shown by the fact that they are lysed by undiluted phages but not by the test dilutions ordinarily used for typing. The reason for this is undetermined; it is conceivable that an alteration in the duration of the phage-sensitive period of growth occurs similar to that which was postulated by Craigie and Yen in the case of the typhoid bacilli.[7] It is possible that certain untypable strains carry latent phage which interferes with the action of the phages used in typing. We have examined several untypable cultures and have been unable to demonstrate that they carried phage, but the series is too small to permit any conclusion. Finally, there is need for a more definitive schema for the identification of phage types and of the fine distinctions which exist within the broad subdivisions.

Taking these limitations into consideration, phage typing has proved to be a useful aid in differentiating coagulase-positive staphylococci and in studying the relationships of strains which have been isolated in repeated cultures from the same or different sources. At present the method does not appear to be readily adaptable to use in the routine diagnostic laboratory, but it could be employed profitably in epidemiologic studies on certain staphylococcal infections or in problems which involve the correlation of cultures during the course of an experimental or clinical study.

SUMMARY

A technique for the phage typing of coagulase-positive staphylococci is out-

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lined which is based on methods described by Fisk and by Wilson and Atkinson.

Six broad phage types are recognized which are distinguished by the sensitivity of cultures to distinct groups of phages of lysogenic origin; within each type, cultures may be lysed either by a single phage or by several phages acting in various combinations.

The sensitivity of 539 coagulase-positive strains of staphylococci was determined. Of these, 290 (53.6%) were susceptible to one or more of a series of 25 phages and were assigned to phage types, 4 (0.7%) gave equivocal results and could not be assigned to type, and 245 (44.7%) were not lysed by any phage of the series.

Examples are given of the use of phage typing in the identification and differentiation of staphylococci.

Just as this paper was about to be submitted for publication an article by Williams and Rippon on bacteriophage typing of *Staph. aureus* appeared in *The Journal of Hygiene* (1952, **50**: 320–353). The reader is referred to this excellent report for a detailed discussion of the techniques and problems of the phage typing of the staphylococci. It may be noted here that the observations described above conform in a number of respects to those reported by Williams and Rippon.

HEPATITIS IN LABORATORY MICE

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The numerous attempts to transmit hepatitis virus A or B to a variety of experimental animals have been either unsuccessful or, in those instances where transmission was reported to have been accomplished, the results have not been confirmed. Although the spontaneous appearance of filtrable agents causing hepatitis in mice[1–4] and puppies[5] has been observed, there is no recognized relationship between such agents and those producing hepatitis in man. A transmissible form of hepatitis was also described in mice[6] and rats[7] treated with urethane and cortisone, respectively, and subsequently inoculated with potentially infectious materials obtained from patients with hepatitis. Although the exact nature of the disease which appeared in mice or rats is not clearly defined, there is no evidence, as yet, to indicate that it is related to human hepatitis.

Variations in susceptibility to certain infectious agents or transmissible tumors have been described in inbred strains of mice[8,9] and in other strains or species following treatment with cortisone,[10–13] urethane,[14] and roentgen radiation.[15] Because of these observations, attempts were made in this laboratory to infect various strains of mice with hepatitis virus alone or in conjunction with the administration of these agents,*

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* The cortisone was furnished through the courtesy of Merck & Company, Inc., Rahway, N. J.

and it is the purpose of this paper to describe the results of these experiments.

METHODS AND MATERIALS

Mice.—Four inbred strains of mice, dba (subline 1), C3H (Jax), C57 (brown-cd), and A albino C (C), from the Jackson Memorial Laboratory, Bar Harbor, Maine, and two strains of albino mice, CFW and Bagg, obtained from Carworth Farms and Mrs. Flora O'Grady, respectively, were used. All the animals were between 3 and 4 weeks of age. Groups of 4 to 9 mice were used for each material tested.

Infectious materials.—Among the difficulties frequently encountered in attempting to adapt hepatitis virus to laboratory animals is the inability to be sure that the materials used for inoculums are infectious. It is quite possible that some of the presumably infectious materials used in these experiments did not contain virus, although the resistance of the agent and the time of collection of the specimens would suggest that the majority of them were infectious.

Acute phase serum (6th to 10th day of disease) was obtained from 30 patients with hepatitis in a United States military hospital in Germany. Since there is no method of differentiating between homologous serum hepatitis and the naturally occurring disease, it is not known how many cases of the former were in the group. It is of interest, however, that one of the serums produced hepatitis in volunteers after a long incubation period,[16] suggesting that at least one patient had this form of disease. The serums were frozen at once and kept at dry-ice box temperature for periods ranging from 2 to 6 months before use, with the exception of one serum, which was stored 18 months. The normal serums used for control purposes in various experiments were also obtained from American soldiers in Germany and stored under similar conditions.

Potentially infectious feces were obtained in the acute phase of disease (3rd to 15th day) from 4 patients. One of these specimens had previously produced hepatitis in volunteers after a short incubation period.[17] The feces were frozen at once and kept at dry-ice box temperature for periods ranging from 6 months to 8 years before use.

Serums for serologic tests.—Paired specimens of serums were obtained during the acute and convalescent phases of hepatitis in 35 patients in Germany. They, as well as the normal control serums, were frozen at once and stored at dry-ice box temperature for periods of 3 to 6 months. Immediately before use, the serums were heated to a temperature of 56 C for 30 minutes. A number of serums containing increased amounts of bilirubin were anticomplementary, and it was found that they could be made usable by centrifugation at a speed of 18,000 r.p.m. in an angle head centrifuge for 30 minutes.

Antigens for complement fixation.—Antigens were prepared from murine ascitic fluid and from the livers of normal and infected mice. Ascitic fluid was removed from the mice and either frozen or allowed to stand overnight in the ice box. Before use, it was centrifuged for 1 hour at 1800 r.p.m. and diluted 1:4 for the test. Antigens were made from murine liver by centrifuging a 10% suspension of liver in sterile isotonic solution of sodium chloride for 30 minutes at 5000 r.p.m. in an angle head centrifuge and diluting the supernate to 1:4 for the test.

EXPERIMENTAL

Effect of cortisone and ACTH

Thirty acute phase hepatis serums were inoculated intraperitoneally into thirty groups of each of four inbred strains of mice (dba, C3H, C57, and Cloudman albino) and also into similar groups of each strain following therapy with cortisone or ACTH. Groups of each strain of mice were also treated with cortisone or ACTH and observed as controls. Cortisone or ACTH was administered to the inoculated and control groups according to the following different schedules: cortisone in amounts of 2.5 or 5.0 mg subcutaneously in a single dose; cortisone in amounts of 0.3 to 0.5 mg daily subcutaneously for 4 to 9 days; ACTH in amounts of 0.25 to 0.4 mg subcutaneously in 2 doses 8 hours apart for 4 to 6 days.

Twenty-eight of the serums were inoculated into mice which received only

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a single injection of 2.5 to 5.0 mg of cortisone. These animals and their controls were observed for 35 days; they were then sacrificed, and specimens of liver were obtained for histologic study.

Two of the serums were inoculated into groups of mice 3 days after the cessation of a course of 6 to 9 days of therapy with cortisone or ACTH. During the 2nd, 3rd and 4th weeks after inoculation, 2 serial blind passages (10% suspension of liver and spleen in sterile isotonic saline) were made from these animals and their controls into comparable groups of the same strains of mice. Specimens of liver of animals of each group were obtained for examination on the last day of therapy with cortisone or ACTH, approximately 1 week after inoculation with serum or passage material, and at varying intervals thereafter up to 5 weeks after inoculation.

No recognizable evidence of adaptation of hepatitis virus was found in any of the 4 strains of mice which received potentially infectious serums or the serums in conjunction with therapy with cortisone or ACTH. The animals inoculated with serum or serum-passage material remained well throughout the period of observation, and there were no demonstrable histologic changes in their livers. There was a wide variability in the response of the different strains of mice as well as of different lots of the same strain to varying dosages of cortisone and, in general, the C3H strain tolerated the largest dosages. Fifty percent or more of the dba (0.5 mg cortisone for 6 days) and C57 and Cloudman albino (0.5 mg cortisone for 9 days) died, while approximately 30% of the C3H (0.5 mg cortisone for 9 days) died. None of the mice receiving 0.3 mg of cortisone daily for 4 days or those receiving varying doses of ACTH revealed any evidence of deleterious effect. The C3H strain tolerated a single dose of 5.0 mg of cortisone reasonably well, with a mortality of 25%, while the mortality of the other 3 strains (dba, C57 and Cloudman albino) was 60% or greater. The latter 3 strains, however, tolerated a single dose of 2.5 mg of cortisone reasonably well, with a mortality rate of 25%. Histologic alterations occurred in the livers of certain animals which received the larger doses of cortisone, with or without acute phase serum, and consisted of a wide range of severity ranging from fatty metamorphosis to hemorrhagic necrosis.

Effect of urethane

The description of Jordan and Mirick[6] of a transmissible form of hepatitis occurring in a strain of albino mice (Bagg) treated with urethane and inoculated with presumably infectious material raised the question as to whether this might be accomplished in other strains of mice.

Groups of one inbred strain (dba) and 2 albino strains (CFW and Bagg) were given drinking water containing 0.1% urethane for 7 days and then inoculated intraperitoneally or fed with serums or feces obtained from 17 patients in the acute phase of viral hepatitis. Eleven control groups were inoculated or fed with serum or feces from normal persons or with sterile isotonic saline. Some control groups were kept in the same quarters with the animals inoculated with potentially infectious materials, and others were isolated. All animals continued to receive drinking water containing 0.1% urethane, with the exception that the urethane was withheld from the drinking water for 3 days, during which those undergoing oral inoculation emptied their water bottles. Two serial blind passages (10% suspen-

TABLE 1.—*The occurrence of hepatitis in three strains of mice treated with 0.1% urethane and inoculated with potentially infectious materials or with control materials.*

Exp. no.	Groups of mice	Occurrence of hepatitis and strains of mice used		
		Original inoculation	Passage 1	Passage 2
I	6	Present in one control (CFW)*	Present in all groups including controls (CFW)	Present in all groups including controls (Bagg)†
II	12	Present in some controls and some groups inoculated with infectious materials (Bagg)	Present in all groups including controls (Bagg)	Present in all groups including controls (Bagg)
III	10	Absent in all groups including controls (dba)‡	Absent in all groups including controls (dba)	Absent in all groups including controls (dba)

* CFW, albinos obtained from Carworth Farms.
† Bagg, albinos obtained from Mrs. Flora O'Grady.
‡ dba, obtained from Jackson Memorial Laboratory.

sion of liver and spleen in sterile isotonic saline) were made 3 to 4 weeks after inoculation into similar groups of mice prepared with 0.1% urethane. Specimens of liver were examined from animals of each group at the time of passage.

A comparison of the results found in the 3 strains of mice is recorded in table 1. Ascites and/or hepatomegaly and splenomegaly with focal infiltration or necrosis of the liver occurred in the originally inoculated mice and their subsequent passages in the 2 albino strains but not in the inbred strain (dba).

In experiment I all but 3 of the mice originally inoculated survived and were sacrificed 22 days after inoculation. Ascites was present in one of the control (saline) animals, and this was not included in the passage material. During the 4th week after the 1st blind passage, ascites and/or hepatomegaly and splenomegaly appeared in both strains of mice from all 6 groups (including the controls). Varying degrees of mononuclear cellular infiltration and focal necrosis of the liver were found. Similar gross and microscopic alterations were found in animals from each group of the 2nd blind passage.

In experiment II, 12 out of the 72 mice originally inoculated died during the 27-day period of observation. Ascites and mild cellular infiltration of the liver occurred in occasional animals among the both sets of control groups as well as among the groups inoculated with potentially infectious serum. There were no deaths in the 1st passage and only 6 in the second; however, in both the 1st and 2nd passage animals, ascites and/or hepatomegaly and splenomegaly, with varying degrees of hepatic cellular infiltration and focal necrosis, appeared in members of all groups.

In experiment III all but 1 of the 120 mice inoculated originally and in the 2 subsequent passages survived. One mouse had moderate enlargement of the spleen but neither hepatomegaly nor ascites was found. Sections of the liver of each animal were examined and were found to be normal.

Effect of roentgen radiation

Attempts were made to increase the susceptibility of mice to hepatitis virus by treating them with roentgen radiation before and after inoculation with potentially infectious materials. Because of the difference in response to inoculation of such materials as well as control materials in different strains of mice treated with urethane, it was believed desirable to compare the response of CFW and Bagg strains and an inbred

TABLE 2.—*The occurrence of hepatitis in three strains of mice treated with roentgen radiation and inoculated with potentially infectious materials or with control materials.*

Exp. no.	Groups of mice	Occurrence of hepatitis and strains of mice used			
		Original inoculation	Passage 1	Passage 2	Passage 3
IV	6	Absent in all groups (CFW)*	Present in 2 groups including controls (Bagg)†	Present in 1 group, controls negative (Bagg)	Present in 5 groups, controls negative (Bagg)
V	32	Absent in all groups including controls (dba)‡	Absent in all groups including controls (dba)	Absent in all groups including controls (dba)	

*, †, ‡ See table 1.

(dba) strain of mice under such circumstances.

Twenty-six groups of mice (albino or dba) were irradiated in amount of 75 r over both the dorsal and ventral surfaces for 4 consecutive days and then inoculated. Nineteen groups received acute phase hepatitis serum or feces intraperitoneally or orally, and 7 control groups received normal serum or feces or saline. A single irradiation in amount of 75 r over both the dorsal and ventral surfaces was carried out again 8 days after the last exposure. Twelve other groups of mice (dba) were left untreated but were similarly inoculated with both normal and potentially infectious serum or feces. All control animals (in both the irradiated and nonirradiated groups) were isolated from those receiving potentially infectious materials. Two or three serial blind passages of a 10% suspension of liver and spleen in sterile isotonic saline were made at 21- to 28-day intervals to untreated mice and to mice prepared with irradiation in a similar manner, with the exception that the initial course of therapy was diminished to 3 days in the dba strain because of apparent ill effects from radiation.

A comparison of the results found in 2 strains of mice is recorded in table 2. Ascites and/or hepatomegaly and splenomegaly with focal infiltration or necrosis of the liver occurred in all 3 passages in the albino mice but not in the inbred strain (dba).

In experiment IV there was a wide variability in response to the roentgen radiation. Only 3 of the 36 mice (CFW) originally inoculated sickened and died, but all of the 1st passage mice (Bagg) withstood irradiation poorly, appearing sick, and 8 died. In the 2nd and 3rd passages, the mice appeared healthier, although there were 3 and 4 deaths, respectively, in each passage. Hepatomegaly, splenomegaly and ascites, with histologic alterations in the liver characterized by diffuse and focal mononuclear cellular infiltration and focal necrosis, appeared in the 1st passage in mice from 2 different groups, including the controls. These lesions increased in frequency and severity so that, in the 3rd passage, members of all 5 groups inoculated with serum-passage material had hepatitis, although both the 2nd and 3rd passages of the controls were negative.

In experiment V the 12 groups of untreated animals originally inoculated and their subsequent passages remained well during the period of observation. Examination of the livers of all animals revealed no histologic alterations from normal. Following the original inoculation of the 20 groups treated with roentgen radiation, there were 23 deaths. These occurred in animals receiving both normal and potentially icterogenic materials, although the preponderance of deaths was among those mice fed

potentially icterogenic feces. Cultures made from the livers of such animals revealed *Escherichia coli* and *Staphylococcus aureus*. During the 1st passage there were no deaths, and in the 2nd passage 11 deaths occurred. Examination of the livers revealed no recognizable evidence of adaptation of hepatitis virus, and the only histologic alteration was the rare occurrence of slight mononuclear cellular infiltration (in 2 specimens in the original group, in 4 specimens in the 1st passage, and in 1 specimen in the second passage).

Nature of disease in mice

The disease found in the 2 strains of albino mice, following treatment with urethane or roentgen radiation and inoculation with control materials as well as with potentially infectious materials, was characterized by hepatomegaly, splenomegaly, and ascites. Focal infiltration of the liver with round cells and varying degrees of focal necrosis occurred; reticulo-endothelial hyperplasia of the spleen was frequently present. Death was uncommon and jaundice was rare. The time of appearance of ascites varied from 14 to 31 days after inoculation, although its occurrence was irregular and certain large lots (100) of Bagg albino mice failed to develop a sufficient amount to be demonstrable. However, the presence or absence of ascites was no indication as to the degree of involvement of the liver, and severe focal infiltration or necrosis was often found in mice without ascites. The amount of ascites was usually not great, although as much as 12 to 15 ml were found occasionally. Cultures of ascitic fluid and the liver were bacteriologically sterile, and stained smears of the peritoneal fluid revealed large numbers of round cells (both lymphocytes and mononuclear cells). Giemsa stains of such smears revealed no parasites or inclusion bodies. The disease was transmissible to 4 inbred strains of mice, dba (subline 1), C3H (Jax), C57 (brown cd), A albino C (C), by intraperitoneal inoculation of ascitic fluid or a suspension of liver and spleen. The agent which produced the disease failed to pass a Seitz EK filter, and infectivity was destroyed by heating to 56 C for 30 minutes.

Serologic tests

Although the disease which appeared in the albino mice was present in the controls as well as in those inoculated with potentially infectious materials, an attempt was made to determine any possible serologic relationship between it and human hepatitis.

Paired acute and convalescent phase serums from 35 patients with hepatitis and serums from 12 normal persons were tested for complement-fixing antibody against antigens prepared from normal mouse liver and from ascitic fluid and liver from infected mice. All of the normal serums and all but 2 of the serums of 35 patients with hepatitis were negative. In one patient, complement fixation was present with antigens made from ascitic fluid and normal and infected mouse liver in a titer of 1:16 of serum on the 10th day of disease and negative on the 26th day. This patient was being treated for primary syphilis on the 10th day of his hepatitis. In the 2nd patient, the titer of the serum was 1:16 against all 3 antigens on the 10th day of disease and persisted in this titer to the 54th day, the last time tested. The reactions in the serums of both patients were interpreted to be nonspecific.

COMMENT

Although the attempts to adapt hepatitis virus to four inbred strains of

mice (whether or not they were treated with cortisone or ACTH) were unsuccessful, the opportunity to examine large numbers of specimens of liver of animals injected with human serum or serum-passage material provided a good background for comparison with the results obtained in the strains of mice used in the experiments with urethane and roentgen radiation.

Two different strains of albino mice (CFW and Bagg) were treated with urethane and roentgen radiation; they were subsequently inoculated with potentially icterogenic materials or control materials. There appeared a form of hepatitis in either the originally inoculated animals or after the first passage, in the controls as well as in the animals receiving potentially icterogenic materials. Since in all but one experiment (exp. I) the control mice were isolated in different rooms, the possibility of cross infection was reduced to a minimum. The incidence of disease was far more irregular in the animals treated with roentgen radiation than in those which received urethane, probably explaining its disappearance among the irradiated controls after the first passage. In contrast, similar attempts to produce hepatitis in a single inbred strain (dba) of mice were unsuccessful. These facts, in addition to the failure to demonstrate by complement fixation any serologic relationship between antigens prepared from mice with hepatitis and serums from patients with hepatitis, indicate that an agent inherent in the two strains of albino mice was activated and passed, producing a disease which is unrelated to human hepatitis. In support of this concept is the fact that in the course of other experiments members of five out of ten groups of albino (Bagg) mice inoculated with ten control serums were found to have varying degrees of hepatitis indistinguishable histologically from the lesions found in the mice in the experiments described here. In contrast, examination of the livers of large numbers of four inbred strains, dba, C3H (Jax), C57 brown, and A albino C (C), following the injection of human serums revealed only rarely slight degrees of cellular infiltration.

No effort was made to define closely the causative agent of this transmissible form of hepatitis in mice, although its failure to resist a temperature of 56 C or to pass a Seitz EK filter differentiates it from human hepatitis virus. Attempts to demonstrate parasites or inclusion bodies in the peritoneal fluid or tissues were unsuccessful and infectious materials were bacteriologically sterile. The disease is clinically similar, and the lesions in the liver consisting of varying degrees of cellular infiltration and focal necrosis are histologically indistinguishable from those described in mice by Jordan and Mirick,[6] but no attempt was made to determine any possible relationship between the two diseases.

SUMMARY

1. Attempts to adapt human hepatitis virus to various strains of mice with or without treatment with cortisone, ACTH, urethane, or roentgen radiation were apparently unsuccessful.

2. In the course of experiments with two strains of albino mice treated with urethane or roentgen radiation, a transmissible form of hepatitis was produced in control animals as well as in those inoculated with presumably infectious material.

3. The available evidence indicates that this disease was inherent in these strains of mice and unrelated to human hepatitis.

TUBERCLE BACILLUS LIPOPOLYSACCHARIDE AS ADJUVANT IN THE PRODUCTION OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN RABBITS

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The recent researches of Raffel[1,2] and Choucroun[3] have provided strong support for the belief that the capacity of the tubercle bacillus to produce the tuberculin type of sensitivity depends on its content of a certain lipopolysaccharide. This is found in the "purified wax" fraction of the organism, and consists primarily of a polysaccharide ester of mycolic acid.[2–4] The lipopolysaccharide is not itself the antigen in this sensitization process but serves to render antigenic any appropriate material present at the same injection site. Studies of the experimental allergic encephalomyelitis produced by injection of homologous nervous tissue plus Freund's adjuvant into laboratory animals have indicated that a tuberculin type of sensitivity to homologous nervous tissue is present in such animals and is approximately parallel in intensity to the encephalomyelitis.[5] One may suppose that the effect of the tubercle bacilli in the adjuvant in producing this sensitivity, and perhaps in producing the encephalomyelitis, is due to their content of the lipopolysaccharide in question. The experiments to be presented show that the lipopolysaccharide can indeed replace the tubercle bacilli used in the adjuvant. Furthermore, they provide support for the conclusions already reached, both as to the effect of the wax in producing the tuberculin type of sensitivity and as to the nature of the sensitivity associated with experimental allergic encephalomyelitis.

EXPERIMENTAL

Methods.—Antigenic mixtures were prepared by emulsifying normal rabbit spinal cord and Bayol F (a bland mineral oil) with samples of purified wax, with Pmko, and with H37RA lipopolysaccharide* in the proportions indicated be-

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1. Raffel, S. 1946, The relationship of acquired resistance, allergy, antibodies and tissue reactivities to the components of the tubercle bacillus. Am. Rev. Tuberc. **54**: 564–573.
2. Raffel, S. 1950, Chemical factors involved in the induction of infectious allergy. Experientia, **6**: 410–419.
3. Choucroun, N. 1949, The role of the lipocarbohydrate of the tubercle bacillus in hypersensitivity, acid-fastness, and acquired resistance. Tuberculology, **11**: 25–30
4. Asselineau, J. and Lederer, E. 1950, Sur des differences chimiques entre des souches virulentes et nonvirulentes de mycobacterium tuberculosis. Compt. rend. Acad. d. sc. **230**: 142–144.
5. Waksman, B. H. and Morrison, L. R. 1951, Tuberculin type sensitivity to spinal cord antigen in rabbits with isoallergic encephalomyelitis. J. Immunol. **66**: 421–444.

* We wish to acknowledge Dr. Choucroun's generosity in providing us with a protein-free sample of purified lipopolysaccharide designated "Pmko." For a complete description of its preparation and properties, the reader is referred to her publications.[3] We are likewise much indebted to Dr. Raffel for supplying a preparation of "purified wax,"[1] together with a sample of lipopolysaccharide, H37RA, prepared originally by Dr. Lederer by fractional elution of purified wax constituents from alumina columns.[4] In view of the similarity of the results obtained with these materials, it is worth noting that the Pmko is obtained by an entirely different fractionation technique from that used in preparing the purified wax.

low. Rabbits weighing approximately 2.5 kg were given a single intradermal inoculation of about 0.05 ml of one of these mixtures into each of the four footpads and were examined daily for signs of neurological disease. Encephalomyelitis, when it appeared, was graded clinically as in previous reports from this laboratory:[5] complete paraplegia or death within 5 days, ++++; the same in more than 5 days, +++; moderate disease, ++; and minimal symptoms, +. On the 14th day, all animals were tested by intradermal injections (0.1 ml) of 10% normal rabbit cord in saline and of a suspension of tubercle bacilli in saline (0.2 mg/ml) into the shaved flank. The skin reactions were graded as in earlier studies,[5] with +++ representing indurated, erythematous reactions greater than 10 mm in diameter, ++ representing reactions between 5 and 10 mm, and + those less than 5 mm. Serum samples, taken on the 14th day and preserved at −15 C, were tested for complement-fixing antibody against nervous tissue by the technique of Lumsden et al,[6] with the use of 0.05% rabbit spinal cord as antigen. As the serums were not anticomplementary (except occasionally in the lowest dilution tested), titers of antibody are reported in the conventional way. The tissues from all experimental animals were subjected to microscopic examination, and the intensity of the encephalomyelitis was graded according to the number of perivascular lesions and the proportion of the total circumference involved by the subpial lesion in transverse sections of spinal cord at three levels. Spinal cord was chosen, because in these and in other experiments, the pathologic changes here were considered representative of the disease process as a whole and appeared to be responsible for the majority of the clinical signs.

Results.—A first experiment with the purified wax sample was unsuccessful. Encephalomyelitis failed to appear in any of 5 rabbits injected with a mixture containing 3 g normal rabbit cord, 20 mg purified wax, and 5 ml Bayol F. Further experiments, however, with the same preparation of purified wax and with the more highly purified materials, did result in the production of encephalomyelitis. Each of the three preparations was used to make up an antigenic mixture as shown in table 1. Each mixture was injected into a group of 5 rabbits.

The experimental results are summarized in table 2. Clear-cut encephalomyelitis, entirely similar to that seen in experiments with whole tubercle

6. Lumsden, C. E., Kabat, E. A., Wolf, A. and Bezer, A. E. 1950, Studies on acute disseminated encephalomyelitis produced experimentally in rhesus monkeys. V. Complement-fixing antibodies. J. Exper. Med. **92**: 253–270.

TABLE 1.—*Antigenic mixtures used to produce encephalomyelitis in rabbits.*

Adjuvant		Normal rabbit cord	Bayol F
Purified wax	27.5 mg	1.5 g	2.2 ml
Pmko	22.5 mg	1.5 g	2.2 ml
H37RA lipopolysaccharide	23.5 mg	1.5 g	2.2 ml

TABLE 2.—*Relation of encephalomyelitis to skin sensitivity and serum antibody titer.*

Adjuvant	Rabbit	Skin sensitivity to:*		Antibody titer*	Clinical disease		Histologic lesions
		Cord	Tbc		Onset	Degree	
					day		
Purified wax	85	+++	0	120	18	++++	++++
	86	0	0	120	0	−	0
	87	++	+	240	16	+++	++
	88	+ to ++	0	120	19	++++	+++
	89	++	0	240	16	++++	++++
Pmko	90	0	0	120	0	−	0
	91	++	0	0	17	++	+++
	92†	−	−	30	−	−	0
	93	±	0	30	0	−	0
	94	±	±	60	0	−	0
H37RA lipopolysaccharide	46	+ to ++	++	120	0	−	0
	47	++	++	240	13	±	0
	48	++	+ to ++	480	11	++	+++
	49	±	++	240	0	−	0
	50	+	++ to +++	240	24	++	++

* On 14th day. Skin tests evaluated at 72 hours.
† Died from bleeding on 14th day.

FIG. 1.—Skin reaction to 0.1 ml of 10% normal rabbit cord in rabbit #91. Test performed on 14th day and photographed on 19th day. Disease onset on 17th day. Indurated and erythematous area is approximately 12 mm in diameter.

bacilli,[6,7] was observed in all three experimental groups. The average day of onset was the 17th, almost exactly that observed in rabbits inoculated with the usual mixture containing Freund's adjuvant.[5] There was a good correlation of clinical and histologic findings. It is of interest that the positive reactions to skin tests with rabbit cord were in close agreement with the occurrence of encephalomyelitis. An apparent exception was rabbit #50, but here the skin test was done 10 days before the development of encephalomyelitis. These skin reactions were clearly of the delayed indurated type, as described in a previous report[5] and illustrated in figure 1. The antibody titers, on the other hand, varied over a considerable range and showed no correlation with the incidence or severity of disease. It would appear that the three preparations tested differed a good deal in their ability to enhance antibody production, as the titers in the H37RA lipopolysaccharide group were uniformly high† and those in the Pmko group almost uniformly low. Here, too, the lack of correlation of antibody formation with disease is seen, for the purified wax gave the most frequent and most severe encephalomyelitis. The skin tests with tubercle bacillus suspension were clearly negative in two of the three experimental groups—those receiving the purified wax and the Pmko.

The histopathological changes in the central nervous system of animals that developed encephalomyelitis were similar to those described in earlier re-

7. Morrison, L. R. 1947, Disseminated encephalomyelitis experimentally produced by the use of homologous antigen. Arch. Neur. Psych. **58**: 391–416.

† This H37RA polysaccharide preparation is particularly active in enhancing antibody formation against tuberculoprotein.[8]

8. Raffel, S. Personal communication.

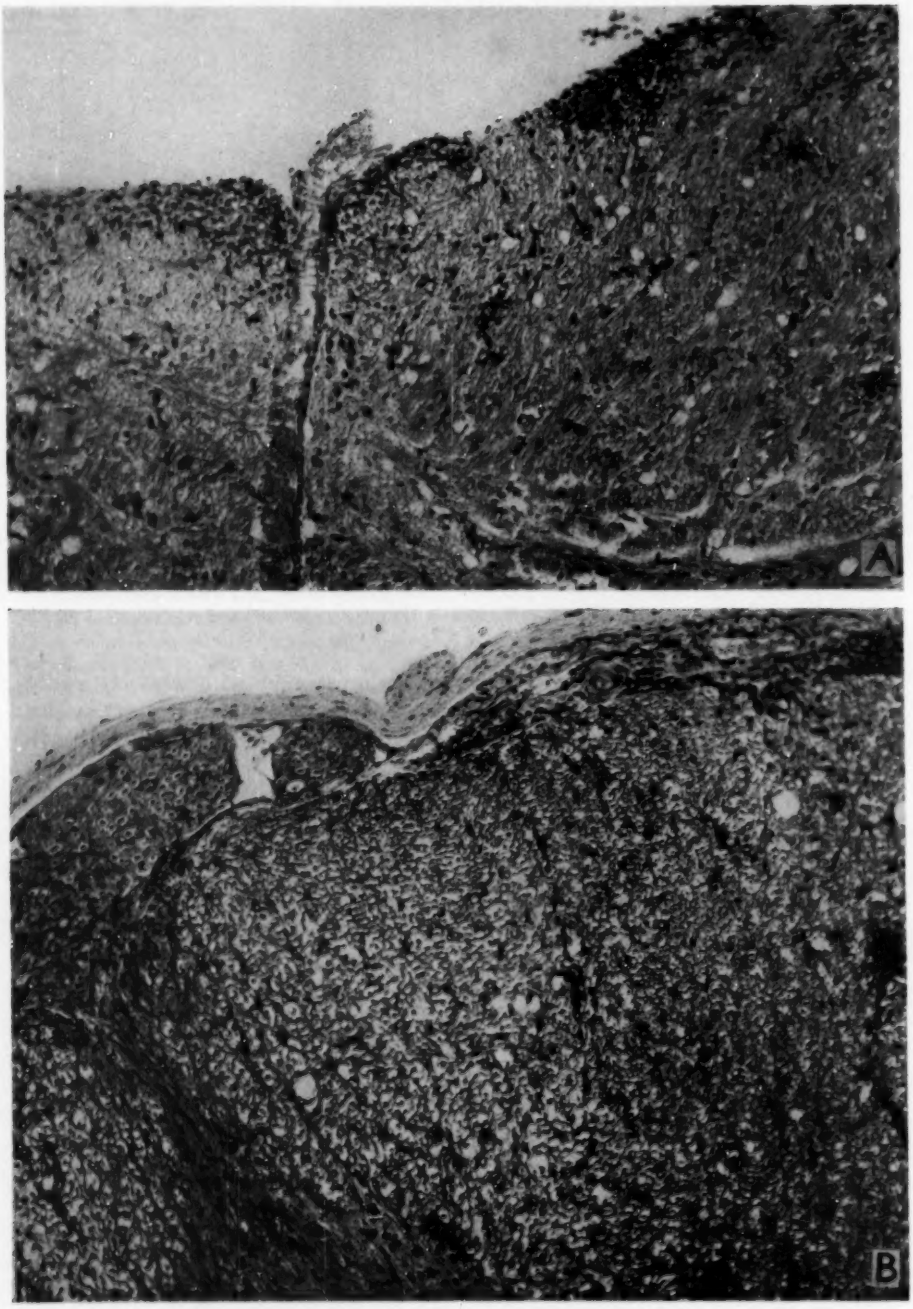


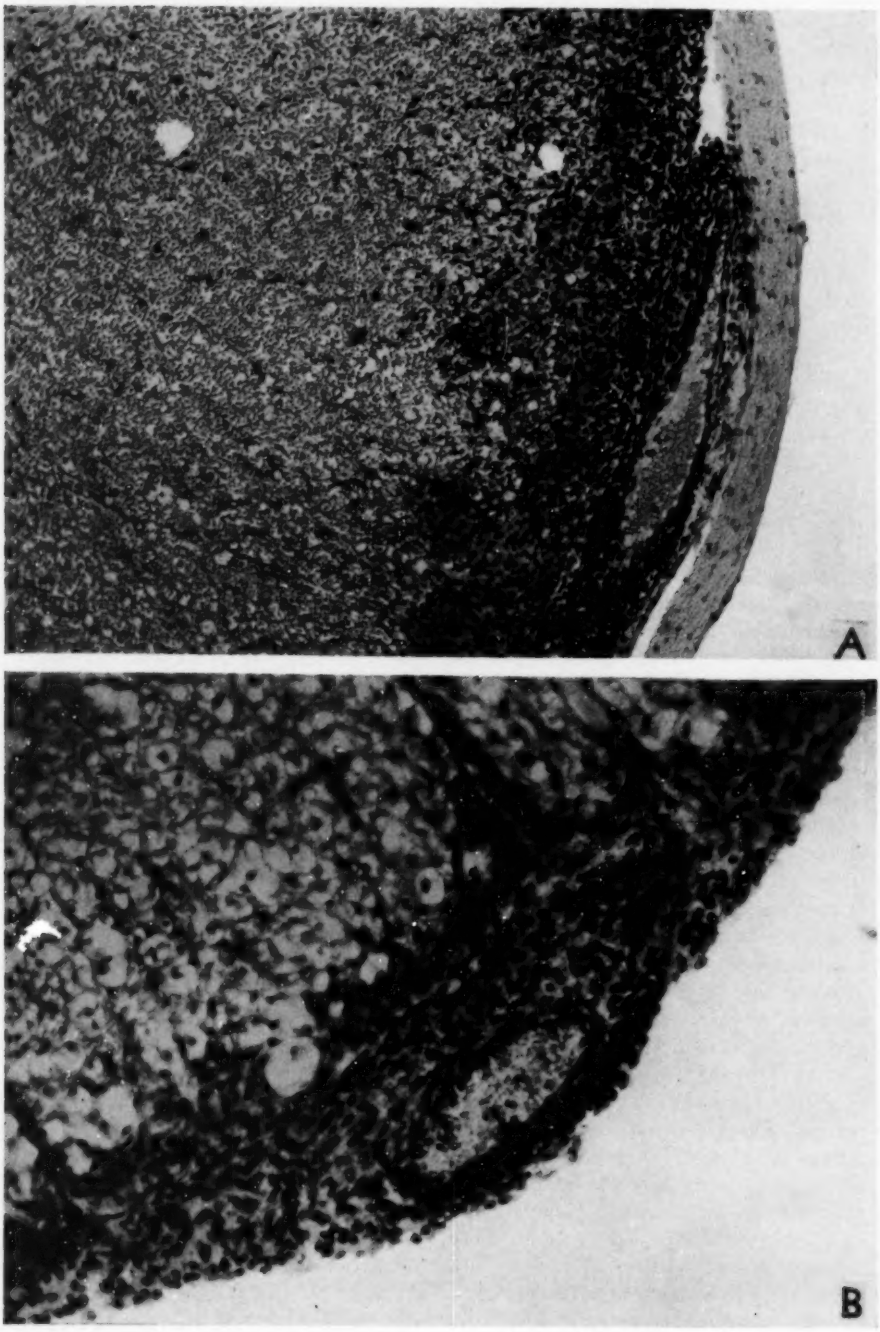
FIG. 2.—Sections of the medulla (A) and spinal cord (B) to show the typical perivascular and subpial foci of microgliacytes in rabbit #88. Hematoxylin and eosin stain, ×50.

ports.[5,7] Infiltrations of lymphocytes, mononuclear leucocytes, and plasmacytoid elements, in the meninges and perivascular spaces, together with microgliacyte proliferation in the subpial and to a slight degree in the paraadventitial regions with destruction of medullated nerve fibers, were the principal features of the process (fig. 2A and B). The lesions were distributed through the spinal cord, medulla, pons, midbrain, diencephalon, and optic nerves and were rarely seen in the cerebrum. In three of of the animals, #48, 50, and 91, there was an unusually intense focalization of the disease in the spinal cord (fig. 3A and B), with extensive degeneration of medullated fibers and phagocytosis of products of degenerated myelin.

DISCUSSION

The essential finding of the present report, that the lipocarbohydrate of the tubercle bacillus is an effective adjuvant in producing allergic encephalomyelitis, constitutes new evidence in favor of the hypothesis already put forward[5] that this disease is associated with a tuberculin type of hypersensitivity. Additional evidence is provided by the good correlation, observed again in the present experiments, of the occurrence of encephalomyelitis with a delayed type of skin reactivity to homologous nervous tissue. The lack of such correspondence with the formation of humoral antibodies to whole rabbit nervous tissue is another point in support of this theory. There was, however, a noteworthy discrepancy in the amount of material needed to produce the observed effects. An antigenic mixture containing 2 mg of whole tubercle bacilli per ml had been effective in previous experiments in producing encephalomyelitis in over half of the rabbits inoculated.[5] Comparable results were obtained only by using a much larger concentration of the purified wax preparation, i.e., 7.5 mg per ml. In the first experiment, when a concentration of about 3 mg per ml was used, no encephalomyelitis was produced. The Pmko, in a concentration of 6 mg per ml, seemed less active than whole organisms and the same was true of Lederer's preparation. Two possible explanations of the lesser potency of these adjuvant materials are that: (1) the active material has been degraded during its preparation; or (2) the active material is not the lipopolysaccharide but some other substance, not yet isolated, which appears in these preparations only as a contaminant. There is also the possibility that the encephalomyelitis, though closely associated with the development of the tuberculin type of sensitization to nervous tissue antigen, may result from some other type of immunologic event brought about by the tubercle bacilli. Unpublished studies in this laboratory have shown that under certain conditions skin reactivity to central nervous system tissue antigens may appear independently of the development of encephalomyelitis. The disease produced in the present experiments could result then from the presence in our test materials of a contaminant that is actually responsible for a different type of adjuvant effect. It should be noted, however, that the three preparations all seemed to be about equally effective in producing both skin reactivity and disease.

The observed effect of the lipopolysaccharide in these experiments supports the hypothesis that this type of material is responsible for the production of hypersensitivity of the delayed or tuberculin type.[1–3] The absence of intact tubercle bacilli from our test preparations, fundamental to the validity of the experiments presented, is attested by the failure of two of the


A
B

three groups of test animals to develop any tuberculin sensitivity, i.e., to react to the skin test with suspensions of tubercle bacilli. The appearance of tuberculin sensitivity in the rabbits receiving the H37RA lipopolysaccharide preparation indicates that this material was not entirely free of tuberculoprotein.‡

The factors governing extent and distribution of encephalomyelitic lesions are not well understood. Though rabbits #48, 50, and 91, which showed a somewhat unusual histological picture, were all animals which had received the more highly purified adjuvants, the differences between the pathologic changes found in them and those present in the rabbits of the purified wax group are comparable to differences which have previously been observed within groups receiving mixtures containing whole tubercle bacilli.

‡ In order to free "purified wax" and its derivative fractions entirely of tuberculoprotein a digestion with proteolytic enzymes must be included in the preparative procedure.[8] This was evidently not done in the case of the H37RA polysaccharide sample studied here.

SUMMARY AND CONCLUSIONS

1. The purified tubercle bacillus wax of Raffel, the lipocarbohydrate "Pmko" of Choucroun, and the lipopolysaccharide isolated by Lederer can effectively replace the tubercle bacillus as adjuvant in the production of experimental allergic encephalomyelitis in rabbits.

2. The quantity of lipocarbohydrate necessary in this experiment was much greater than the effective weight of whole bacilli.

3. Delayed skin reactivity to homologous nervous tissue was produced by antigenic mixtures containing any of these preparations as adjuvant and was well correlated with the development of encephalomyelitis. Reactivity to tubercle bacillus suspension developed only in rabbits receiving Lederer's preparation.

4. Antibody titers measured against whole rabbit spinal cord varied in a random manner in the experiments reported. As in previous experiments, the formation of humoral antibodies to nervous tissue seemed to bear no relationship to the encephalomyelitis.

FIG. 3.—Section of spinal cord of rabbit #91.

In figure A the infiltrating cells around the meningeal vein on the right side of the picture are lymphocytes and histiocytes, and the proliferating cells along the margin of the spinal cord, near the center of the picture, are microgliacytes. In B from the same animal the character of the meningeal exudate is seen at higher magnification. Hematoxylin and eosin stain, ×50 and ×200.

ANTIBACTERIAL EFFECT OF ELEMENTAL SULFUR

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Elemental sulfur is known as an effective agent in combating parasites of plants and animals. Incorporated in ointments, sulfur has been and is still used extensively, particularly for the treatment of parasitic skin diseases. Until recently, however, little has been reported on the effect of this element on bacteria. Lawson and associates (1934–35) and Massey and Snider (1936) found that flowers of sulfur exert an inhibitory action on the growth of tubercle bacilli in concentration of 1:40,000. Lawson (1934) reported, however, that some common pathogenic bacteria are not inhibited by the element. On the other hand, Weld and Gunther (1947) have shown that saturated solutions of sulfur in alcohol and in carbowax, when diluted with broth, are strongly inhibitory to various Gram-positive organisms. They reported that the inhibitory effect is characterized by prolonged bacteriostasis and that no evidence was observed of true bactericidal action with the highest concentrations used. They also found that sulfur had no inhibitory action on a number of Gram-negative organisms. Schuhardt et al (1952) reported recently that sulfur has a toxic effect also against *Brucella abortus*.

EXPERIMENTAL

In studying the effect of sulfur compounds on bacteria, we found that calcium polysulfide added to brain-heart broth in quantities as small as 1 μg per ml, expressed in elemental sulfur liberated by the compound, inhibited the growth of several strains of group A and group C hemolytic streptococcus, *B. abortus*, *Clostridium perfringens*, and other pathogenic bacteria. The inhibition of group A hemolytic streptococcus, strain C203, was observed with an inoculum of 0.01 ml of a 16-hour broth culture, or approximately 1 million organisms per ml of diluted polysulfide.

In all our tests we observed that a precipitate was produced upon the addition of calcium polysulfide to brain-heart broth. Microscopic observation of this precipitate revealed amorphous masses in which particles of an average size of 1 μ were embedded. These particles proved to be elemental sulfur. When the precipitate was centrifuged, washed with distilled water and resuspended in the original volume of fresh sterile broth the growth of streptococci was inhibited under the same conditions as those of the original tubes. On the other hand, the clear broth from which the precipitate was completely removed no longer exerted any inhibitory action on the organisms. It was apparent that practically the entire inhibitory activity originally found for calcium polysulfide was recovered in the precipitate. These results are shown in table 1.

It was indicated to determine what component of the broth reacted with calcium polysulfide to produce the active inhibitory precipitate. Since the culture medium used in the tests contained 0.25% dibasic sodium phosphate, an aqueous solution of the pure salt having the same concentration as that of the broth was prepared. Calcium

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TABLE 1.—*Inhibitory effect of calcium polysulfide, polysulfide-broth precipitate, and polysulfide-phosphate precipitate on group A hemolytic streptococcus, strain C203.*

Compound	Dilution of compound in broth						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Control
Calcium polysulfide (2% solution)*	0	0	0	0	+	+	+
Polysulfide-broth precipitate	0	0	0	0	+	+	+
Polysulfide-Na_2HPO_4 precipitate ($CaHPO_4$-S)†	0	0	0	0	+	+	+
Supernatant broth from polysulfide-broth precipitate	+	+	+	+	+	+	+
Supernatant liquid from polysulfide-phosphate precipitate	+	+	+	+	+	+	+
$CaHPO_4$ gel free of sulfur	+	+	+	+	+	+	+
Small sulfur particles free from $CaHPO_4$ gel	0	0	0	0	+	+	+

0, no growth in broth; +, growth. Inoculum: 1 million organisms per ml.
* This solution diluted to 10^{-4} yields 1 μg/ml of elemental sulfur in broth.
† Prepared by mixing 1 volume 2% polysulfide solution with 9 volumes of 0.25% solution of dibasic sodium phosphate (anhydrous).

polysulfide added to the sodium phosphate solution produced a precipitate similar to that formed in broth. This precipitate also was composed, microscopically, of amorphous masses with embedded particles of sulfur. The reaction taking place can be expressed in the following equation:

$$Na_2HPO_4 + CaS_2 = CaHPO_4 + Na_2S + {}_2S.$$

During the simultaneous formation of calcium phosphate and sulfur, this element is absorbed by the hydrated calcium phosphate gel in the form of very small particles. This precipitate, centrifuged, washed thoroughly with distilled water and suspended in broth, in amounts equivalent to those used for broth and calcium polysulfide solution, showed an inhibitory effect of the same magnitude as that observed with the precipitate obtained from broth and polysulfide. The clear supernatant solution containing sodium sulfide was also tested and proved to be inactive against the organisms. The comparative results shown in table 1 give evidence that the inhibitory effect of calcium polysulfide is due to the direct action of the calcium phosphate-sulfur precipitate.

In order to determine which component of this precipitate was responsible for the inhibitory action, sulfur was removed with solvents (alcohol, ether and carbon disulfide) and the remaining calcium phosphate was tested for inhibitory activity. The results showed that the remaining calcium phosphate was completely inactive. In the course of removing sulfur from the precipitate with solvents, the masses of calcium phosphate became in part dehydrated and lost somewhat their gel-like appearance. To test the effect of calcium phosphate in its original condition, one volume of 2% solution of calcium chloride was added to ten volumes of 0.25% sodium phosphate solution. The precipitate thus obtained showed under the microscope amorphous masses identical to those obtained from calcium polysulfide and sodium phosphate. This precipitate also proved to be completely inactive against the streptococci. It was thus apparent that the inhibitory effect of the calcium phosphate-sulfur precipitate against the organisms was due to the action of the particles of sulfur embedded in the calcium phosphate gel.

To determine whether the calcium phosphate gel had any influence on the antibacterial action of the absorbed sulfur particles, the masses of calcium phosphate were dissolved completely in the broth by careful addition of normal hydrochloric acid. Dilutions were made with the resulting sulfur suspension and the respective tubes were adjusted back to pH 7.4 with normal sodium hydrox-

ide. By this treatment, calcium phosphate remained in solution. Microscopic observation revealed that the particles of sulfur, maintaining the same size of approximately 1 μ, were freely dispersed in the medium. The tests on streptococci showed that the inhibitory activity of this suspension was the same as that of the original calcium phosphate-sulfur precipitate suspension. It was thus established that the antibacterial action of calcium polysulfide on the bacteria is not exerted by the compound itself, but by the elemental sulfur released when this compound is treated with a solution of sodium phosphate.

To test further the activity of elemental sulfur free from calcium phosphate, the element was precipitated from a solution of calcium polysulfide by diluting it with ten volumes of distilled water; also a calcium polysulfide solution was adjusted from pH 10.3 to pH 9.0 with diluted hydrochloric acid, whereby sulfur separated from the solution. Microscopic observation of both precipitates revealed crystalline particles of sulfur of an average diameter of 20 μ. These precipitates of sulfur in relatively large particles were tested against streptococci and showed only slight inhibitory activity (table 2). These results indicated that the antibacterial activity of elemental sulfur depends in a very important degree on the size of the particles.

To explore further the antibacterial activity of elemental sulfur as a function of its particle size, colloidal sulfur was prepared by heating to the boiling point an aqueous solution of sodium tetrathiodiglycollate. The formation of elemental sulfur takes place according to the following equation:

$$NaOOC\text{–}CH_2\text{–}S\text{–}S\text{–}S\text{–}S\text{–}CH_2COONa = NaOOC\text{–}CH_2\text{–}S\text{–}S\text{–}CH_2\text{–}COONa + {}_2S.$$

Immediately after the clear solution of tetrathiodiglycollate became cloudy as a result of the separation of elemental sulfur, the solution was cooled rapidly. A colloidal suspension of the element was thus obtained, the particles of which were approximately 0.25 μ in diameter. This preparation, when tested for inhibitory activity against streptococci, showed complete inhibition through a 1:800,000 dilution. This dilution corresponds to approximately 0.25 μg per ml of free sulfur released by the compound. Sodium dithiodiglycollate resulting from the decomposition of sodium tetrathiodiglycollate proved to be inactive against the organisms. Figure 1 shows the antibacterial effect of sulfur against hemolytic streptococcus, strain C203, in function of the particle size.

The antibacterial activity of highly dispersed sulfur suspensions decreases on standing. At room temperature, most of the activity of the phosphate-sulfur precipitate disappears in one week or ten days, and at 37 C the inactivation takes place in two or three days. This inactivation coincides with the increase in the size of the sulfur particles. The resulting larger particles do not appear, however, under the microscope as clumps formed by the aggregation of small particles. They show a uniform crystalline structure and their growth occurs at the expense of the disappearance of small particles A micro sublimation apparently takes place whereby the smaller particles vaporize faster and condense on larger particles with the formation of large crystals. A similar but accelerated process occurs when the

TABLE 2.—*Inhibitory effect of sulfur particles, average diameter 20 μ, on group A hemolytic streptococcus, strain C203.*

Preparation	Dilution in broth				
	1:10	1:20	1:40	1:80	Control
Sulfur suspension*	0	0	+	+	+
Supernatant liquid	+	+	+	+	+

0, no growth; +, growth.
* Prepared from 2% calcium polysulfide solution with hydrochloric acid.

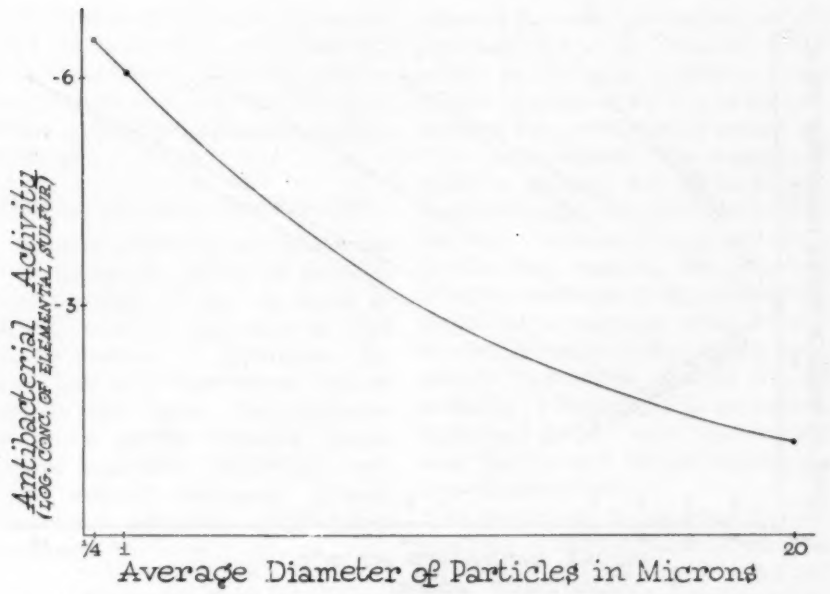


FIG. 1.—Antibacterial effect of elemental sulfur on group A hemolytic streptococcus strain C203, in function of the particle size.

solubility of sulfur in the medium is increased by the addition of alcohol to the suspension, a process which can be observed under the microscope.

On the other hand, sulfur suspensions kept frozen at −20 C for 26 weeks showed no increase in the size of the particles and maintained intact their antibacterial activity.

Effect of −SH compounds on the antibacterial activity of sulfur

Cavallito (1946) and Cavallito and associates (1945) demonstrated that a variety of antibiotic substances are inactivated by cysteine and other −SH compounds. Since sulfur also reacts with −SH compounds, according to the equation

$$R\text{–}SH + S \rightarrow R\text{–}S\text{–}S\text{–}R + H_2S$$

we studied the effect of cysteine, glutathione and thioglycollic acid on the antibacterial action of this element. The −SH compounds were dissolved in distilled water and neutralized to pH 7.0 with normal sodium hydroxide. Each solution was sterilized by filtration through a Seitz filter and added to brain-heart broth in the desired concentration. An 18-hour broth culture of hemolytic streptococcus, strain C203 was used as the test organism. An inoculum of 0.1 ml of a 1:10 dilution of the overnight culture, or approximately 1,000,000 organisms per ml broth was used.

The results have shown that L-cysteine, glutathione and thioglycollic acid incorporated in the culture medium in approximately molecular proportions equivalent to the amount of sulfur added, protect the organisms against the antibacterial action of the element. Similar results were obtained by Schuhardt and associates (1952) with glutathione in neutralizing the toxic effect of sulfur against *Br. abortus*.

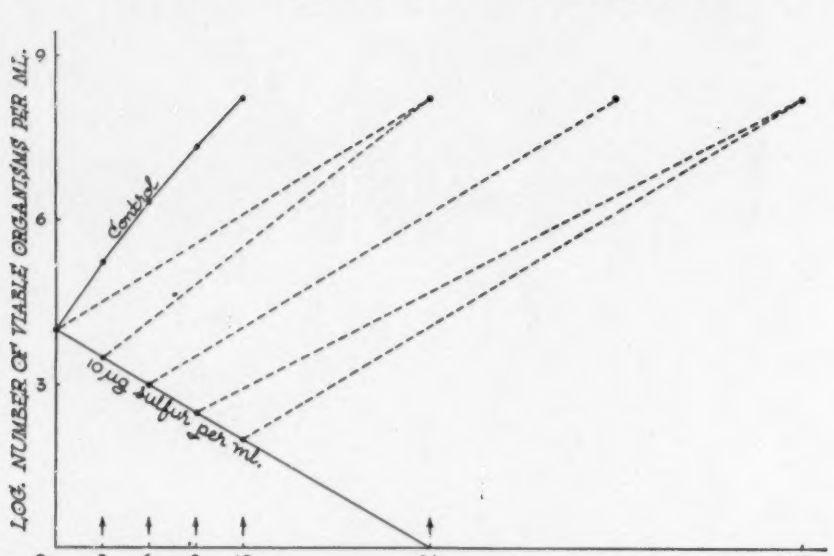


FIG. 2.—Bactericidal action of elemental sulfur on hemolytic streptococcus strain C203. Protective effect of glutathione.

Bactericidal effect of sulfur

The bactericidal effect of elemental sulfur was studied in the following manner: Tubes of brain-heart broth containing freshly prepared sulfur-phosphate precipitate in a dilution of 10 μg sulfur per ml and control tubes without precipitate were inoculated with 0.1 ml of a 1:1000 dilution of a 16-hour broth culture of hemolytic streptococcus, strain C203. This inoculum represented approximately 10,000 organisms per ml of diluted precipitate. Immediately, and again at 3-hour intervals up to 24 hours of incubation at 37 C, samples were subcultured into brain-heart broth and into poured blood agar plates to obtain colony counts. Care was taken that the amount transferred to the subcultures was not sufficient to cause an inhibitory effect. As figure 2 shows, the sulfur-containing cultures showed a gradual decrease in number of organisms until at 24 hours no viable streptococci remained. The control tubes showed a rapid increase in number of organisms, reaching a peak in growth at 12 hours. Figure 2 also shows the effect of the addition of 1 mg/ml of glutathione to a duplicate series of sulfur-containing tubes at various intervals after inoculation. It is evident that glutathione was effective in protecting the organisms against the toxic action of sulfur when it was added at any time up to and including 12 hours after inoculation. When added at 24 hours no growth occurred, and it was shown that at this time no viable organisms were present.

Development of resistance

Hemolytic streptococcus C203 was passed serially through brain-heart broth containing a sublethal concentration of elemental sulfur as present in the sulfur-phosphate precipitate. After eight serial transfers in a dilution of 1:20,000

and 1:10,000 in which the culture would grow with an inoculum of 0.1 and 0.2 ml of the preceding undiluted culture, the organisms were still inhibited by a dilution of 1:10,000 (using an inoculum of 0.01 ml).

Organisms sensitive to elemental sulfur

The sulfur-phosphate precipitate was active against two strains of group A hemolytic streptococcus, two strains of group C hemolytic streptococcus, type I pneumococcus, *Cl. perfringens*, *Br. abortus* and to a lesser degree against *Staphylococcus aureus*. No inhibition was shown for the following Gram-negative organisms: *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhosa*, *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*.

Mechanism of action

Hemolytic streptococcus suspensions in broth containing various amounts of sulfur-phosphate precipitate were examined under the microscope immediately after inoculation of the organisms, at intervals during incubation, and at the end of the experiments on bactericidal action. No evidence of agglutination or attachment of the organisms to the sulfur particles was observed, indicating that the antibacterial action is not exerted by the direct contact of the microscopic sulfur particles with the bacterial cells. This observation and those described concerning the strong antibacterial effect of highly dispersed sulfur in contrast to the low activity of larger particles, would suggest that the vapor pressure of sulfur might play a decisive role in the mechanism of action of the element. According to Lord Kelvin's equation,

$$\Delta F = RT \ln \frac{p}{P_A} = \frac{2\gamma v}{r},$$

the vapor pressure is higher as the radius of the particles is smaller. In this expression ΔF is the increase of free energy, p the vapor pressure of the particle of radius r, P_A that of the substance in bulk, γ the surface tension and v the molal volume. This equation is valid for solids as well as for liquids. Applied to sulfur, the difference between the vapor pressure of small and large particles and, therefore, the differences in the concentration of molecular sulfur in the culture medium would account for the differences in their antibacterial activity. It would be expected that the solubility of small particles of sulfur is higher and that a critical concentration must be attained for the bactericidal effect to take place.

In attempting to ascertain the dependence of the antibacterial effect on the respective solubility of large and small particles, highly dispersed elemental sulfur in the form of sulfur-phosphate precipitate (average diameter 1 μ) and larger crystals (average diameter 20 μ) of the element were prepared by the procedures described above. Both precipitates were thoroughly washed to eliminate all traces of sulfide and suspended in distilled water. The suspensions were adjusted to pH 7.2 with sodium phosphate and incubated for 2 hours at 37 C with occasional stirring. The suspensions were filtered through ultra fine fritted glass filters and the filtrates reduced by evaporation to small volumes after addition of sodium hydroxide. No traces of sulfides or microscopic sulfur particles could be detected in the filtrates before the addition of alkali. Several tests showed, in effect, that the filtrates from suspensions of small particles contain larger amounts of sulfur dissolved than the filtrates from suspensions of larger particles.

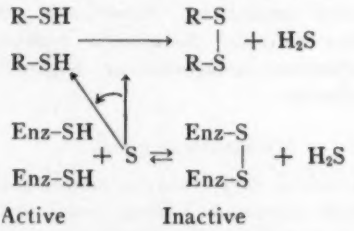
In all these tests, freshly boiled and cooled distilled water was used in the preparation of the suspensions to avoid

possible oxidation of sulfur by dissolved air. It cannot be entirely ruled out, however, that a formation of polythionates might have taken place during the incubation of the suspensions. With the means at our disposal we could not determine quantitatively the concentration of molecularly dispersed sulfur in the aqueous medium. The spectrophotometric method developed by La Mer and Kenyon (1947) should furnish more accurate and specific quantitative data on the solubility of sulfur in an aqueous medium in function of the particle size, pH and temperature. In a series of studies on formation and growth of colloidal sulfur, these authors have established that the critical supersaturation concentration of molecular sulfur S_8 in aqueous solution is approximately 5×10^{-6} g atoms S/1. We do not know at the present time what concentration is attained when highly dispersed sulfur is incubated in the culture medium. Nevertheless, this maximum figure would indicate that the actual bactericidal concentration of elemental sulfur molecularly dispersed in the medium is considerably lower than that of the total sulfur added with the precipitate.

DISCUSSION

Experimental evidence has shown in the last fifteen years (Hellerman, 1937; Rapkine, 1938; Barron and Singer, 1945; Singer and Barron, 1945) that numerous enzyme systems require for their activity the presence of free SH groups. Barron (1949) listed a large number of these enzymes essential for the normal intermediary metabolism of carbohydrates, fats and proteins. These investigations have demonstrated that a variety of toxic agents, including heavy metals, halogenated compounds, oxidants and ultraviolet and X radiations have in common the property of inactivating these enzyme systems either by blockading their SH groups or by transforming them into -S-S- linkages. In most cases the activity of these enzymes can be restored by the addition of glutathione or cysteine.

The reactivity with SH groups and the antibacterial action of elemental sulfur gives further evidence that agents capable of blockading or oxidizing free SH groups will exert a toxic action on the living cell (Libenson, 1945). This action and the protective effect of equivalent amounts of glutathione, cysteine and thioglycollic acid can thus be represented by the following diagram:



The SH compounds would deflect sulfur from inactivating the cellular SH enzymes by removing the element from the system.

The inactivation of an SH enzyme by elemental sulfur has already been demonstrated by Thunberg (1915) and confirmed by Bergstermann and Lummer (1947) with succinic dehydrogenase. Further work is indicated to determine the effect of highly dispersed sulfur on the other SH enzyme systems.

SUMMARY

1. Evidence is presented that elemental sulfur in high degree of dispersion exerts a strong inhibitory action against a number of pathogenic bacteria.

2. This antibacterial effect was observed against several strains of hemolytic streptococcus, *Clostridium perfringens*, type I pneumococcus, *Brucella*

abortus and to a lesser degree against *Staphylococcus aureus*. No inhibitory action was observed against the following Gram-negative organisms: *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhosa*, *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*.

3. Glutathione, L-cysteine and sodium thioglycollate neutralize the antibacterial activity of elemental sulfur.

4. The antibacterial action of small particles of sulfur as contrasted with the low activity of larger particles is explained by the higher vapor pressure exerted by the small particles in the culture medium.

5. The mode of action of sulfur is discussed and it is suggested that the antibacterial effect of the element might be due to the inactivation of the SH enzyme systems of the bacterial cell.

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DIRECTED LEUCOCYTE MIGRATION IN RESPONSE TO INFECTION AND OTHER STIMULI

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Our knowledge of the response of blood leucocytes from normal animals to bacteria and their products has been stimulated in recent years by development of techniques permitting greater understanding and a more quantitative treatment of the process (Martin et al, 1950; Miles and Niven, 1950). The ability of certain organisms to inhibit the migration of leucocytes in vitro has also been correlated in some cases with the production of endotoxins by the organisms (Martin and Chaudhuri, 1952) and with the formation of cell substances apparently associated with the quality of virulence.

The objectives of the present study have been to examine the phenomenon of leucocyte migration in response to brucellae and some of their products, to extend the study to leucocytes derived from animals infected with brucellae and other organisms, and finally to determine whether the leucocytes of infected animals or of animals treated in other ways are inherently affected so far as migration capacity is concerned by changes impressed upon the host or whether the leucocytes are merely indirect indicators of plasma alterations.

METHODS

The general outlines of the method were described by Martin et al (1950). All test tubes, syringes and needles were coated with methyl chlorosilicon (G.E. dri-film 9987) after having been scrupulously cleaned and dried. After coating, the glassware was rinsed 1 hour with distilled water, 1 hour in hot tap water, and ½ hour again in distilled water. The equipment was then soaked for ½ hour in 3% ammonium hydroxide, rinsed in 4 changes of distilled water, dried at 125 C in the air oven, plugged, wrapped, and sterilized in the autoclave.

Slides and cover slips to make the chambers described by Martin et al (1950) were kept in a mixture of 5% nitric acid and 95% sulfuric acid for 1 to 2 hours. They were then rinsed in distilled water and allowed to stand in A. H. Thomas Laboratory cleaning aid for ½ to 1 hour. After a thorough rinse in distilled water the glassware was stored in 95% alcohol.

The microchambers were fashioned after the diagram in the above reference.

Guinea pigs were selected as the experimental animal. The exact amount of heparin which allowed 2 ml of blood to remain liquid while being manipulated and to clot as soon as possible thereafter was determined.

Up to 5 animals were used for each set of data. Four ml of blood per animal were taken by silicon-coated syringes and needles. Two ml of blood were placed in each of 2 coated tubes. The tubes contained also 0.1 mg heparin (containing not less than 6 Toronto units) in 0.1 ml saline. To one tube was added 0.1 ml saline and to the other 0.1 ml saline containing either 6×10^8 organisms or a desired amount of test substance. The tubes were mixed and incubated 20 minutes at 37 C. The mixtures were then dispensed into chilled chambers, using cold pipettes throughout. After sealing the open end of the chamber with paraffin the chambers were centrifuged 5 minutes at 4 C at about 200 G while covered with a liquid, such as chilled Roccal solution. The chambers then were placed at a 10° angle in a 37 C box. After 1 to 2 hours sufficient clotting of the plasma had occurred to begin the readings.

The readings were based on the greatest distance migrated by a field of 25 to 50 leucocytes as observed through the high, dry objective lens. In view of the variation from animal to animal 4 chambers were prepared from each animal and up to 5 animals employed.

In recording the observations and compiling

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the final graphs it was decided to plot the mean value of the set of 4 chambers from each animal against time. The variation in the data of 4 or more animals making up the test and control groups, respectively, reveals itself by the general size of the area covered on the graph. Although the data from different animals may vary considerably, the deviation from the mean distance of the individual chambers of a set of four was remarkably small. In most cases all slides were examined for 6 to 9 hours.

The strains of brucellae used in the study were the virulent PS-3 strain of *Brucella suis*, the relatively avirulent A-19 strain of *Brucella abortus*, and the avirulent Str-d strain of *Brucella militensis* which is dependent upon the presence of presence of streptomycin for growth. All of these strains have been well standardized in earlier studies in these laboratories in terms of their infectivity and other bacteriological characters.

RESULTS

Response of leucocytes of normal guinea pigs.—Orienting experiments showed that virulent strains of the species of brucellae inhibited the migration of guinea pig leucocytes (fig. 1) and that avirulent cells failed to manifest this property.

Two fractions derived from the cells of virulent brucella strains were tested for their ability to retard leucocytic migration. These fractions were the "native antigen" of *Br. suis* (Miles and Pirie, 1939; Silverman and Elberg, 1950) and an antigen very active in detecting cutaneous sensitivity to brucellae infection (Benedict and Elberg, 1953). Only the skin-test antigen inhibited the migration of the leucocytes. A titration of its activity is shown in figure 2. It is of interest that the minimum effective dose of antigen for inhibition of migration corresponds to the actual skin-test dose most effective in guinea pigs for eliciting cutaneous response one week after infection. It is of further interest that the inactivity of avirulent cells was associated with the apparent absence of antigen active in the cutaneous hypersensitivity response. Thus, the hypersensitivity antigen is not only an effective inhibitor of leucocyte migration, but is also associated with the quality of virulence in brucellae.

Response of leucocytes of treated guinea pigs.—A marked change occurred in the leucocytic pattern of response when the leucocytes were derived from animals which had been variously treated.

Animals which had been infected with virulent *Br. suis* for periods of 1 hour to 4 weeks before the blood sample was taken possessed leucocytes in the peripheral blood which were insusceptible to virulent brucellae as far as inhibition

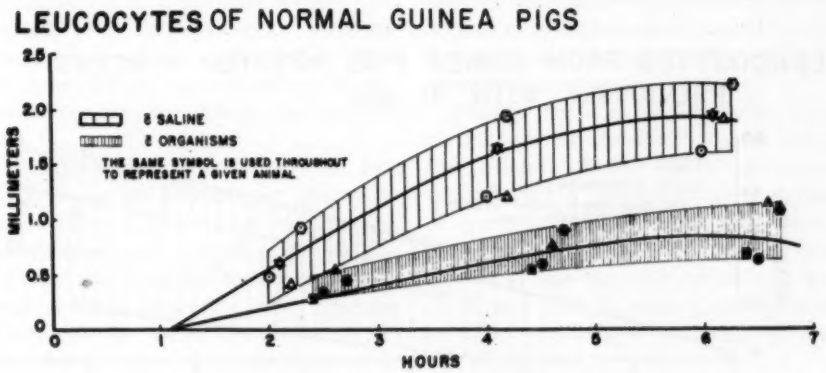

FIG. 1.—Response of leucocytes of normal guinea pigs to virulent *Br. suis*.

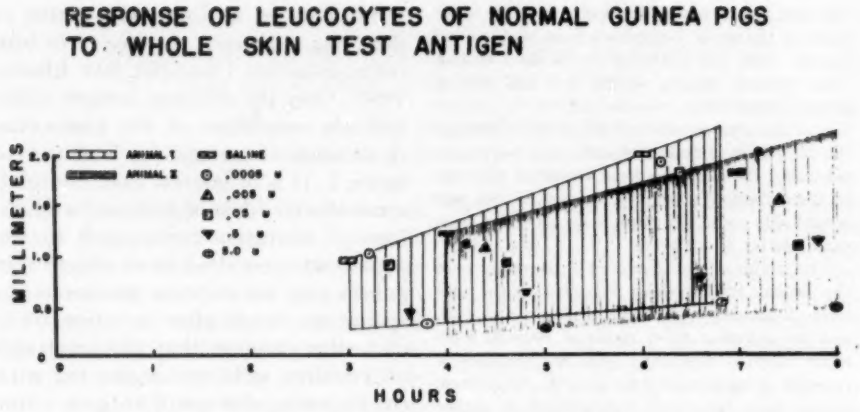

FIG. 2.—Titration of ability of brucella skin-test reagent to inhibit leucocyte migration.

of directed migration is concerned. Representative data are shown in figure 3 for the 4-week period. The congruence of the points for leucocytes exposed either to saline or to virulent cells indicates the inability of the bacterial cells to affect the migration.

To study this phenomenon in more detail, two sets of experiments were performed which pointed to an inherent change having occurred in the leucocytes as a result of injecting the animals with various substances.

The first set of experiments was designed to study the role of the plasma in the reaction. Blood from normal animals and from animals which had been infected at a prior date were obtained in the usual manner, centrifuged and the plasma removed from each tube and saved. The red and white cells from each tube were then washed several times in Tyrode's solution to remove residual plasma. The original plasma was then placed into the heterologous cell mixtures. In this way an exchange was made between the plasma of, for example, normal animals with that from infected animals. The final mixtures contained leucocytes and red cells derived originally from one kind of animal suspended in the plasma from

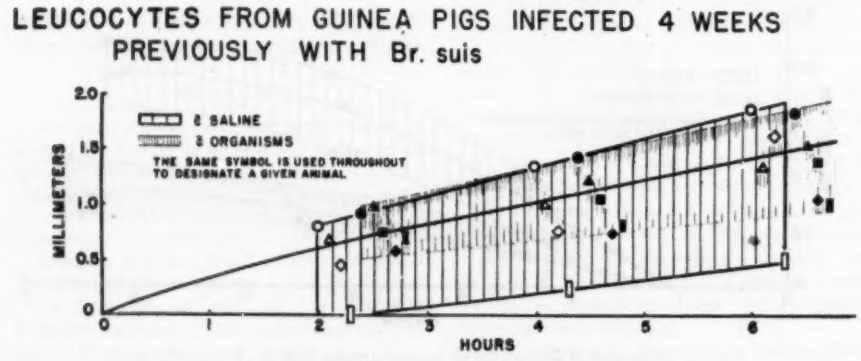

FIG. 3.—Behavior of leucocytes derived from infected animals.

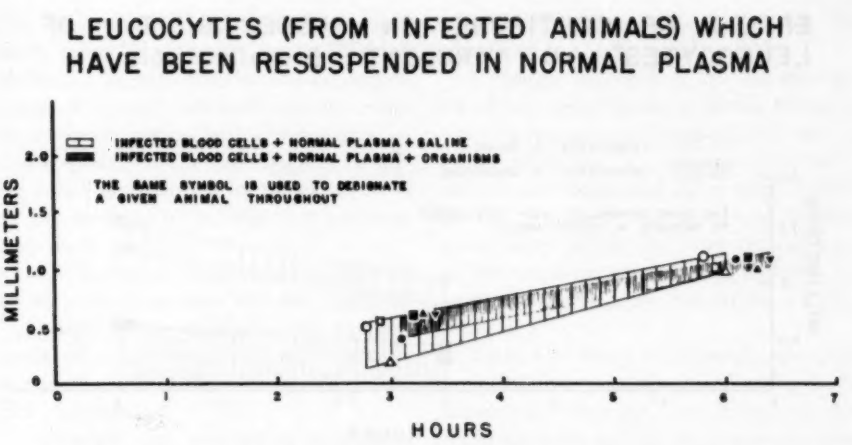

FIG. 4.—Plasma transfer experiment, indicating independence of leucocytes upon plasma.

the other type of animal used in the experiment. The organisms were then added to the mixtures and the chambers prepared. The results shown in figure 4 indicated that leucocytes responded to virulent brucellae in a manner characteristic of those of the animal from which they were originally derived and were not influenced by the plasma of the chamber per se. Thus, leucocytes derived from infected animals and insusceptible to virulent brucellae retained this insusceptibility when resuspended in plasma derived from normal animals. Conversely, leucocytes of animals not previously treated remained fully susceptible to virulent brucellae even when suspended in plasma derived from animals whose leucocytes were insusceptible to the virulent cells.

The second approach to the hypothesis that the leucocyte is inherently responsive and affected by treatment of the whole animals was suggested by the study of the increased ability of macrophages when derived from animals physiologically under the influence of cortisone to support intracellular growth of *Mycobacterium tuberculosis* (Lurie et al, 1951).

Guinea pigs were given 25 mg cortisone subcutaneously each day for 5 days. On the 4th day of cortisone treatment the animals were infected with a virulent strain of *Br. suis*. The animals were bled on the 5th day of treatment. The leucocytes of these animals were completely susceptible to the inhibiting action of *Br. suis* in vitro, in marked contrast to the insusceptibility of leucocytes derived from animals similarly infected but not under cortisone treatment (fig. 5). Cortisone itself did not affect the migration velocity of the leucocytes of animals receiving it alone, for such leucocytes when exposed to saline instead of to organisms migrated at the same rate as did leucocytes derived from untreated animals.

Plasma transfer experiments were carried out as described above, using the plasma derived from cortisone-treated animals to replace in vitro that of normal animals, and vice versa. The behavior of the leucocytes as just described was maintained under these conditions and the drug thus appeared to affect the inherent susceptibility of the leucocyte.

Although the blood of normal guinea pigs contains leucocytes susceptible to

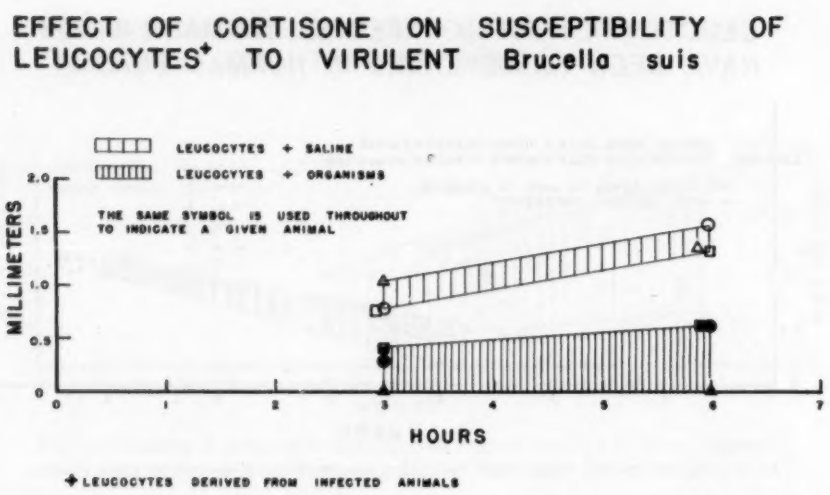

FIG. 5.—Ability of cortisone (25 mg per day for 5 days) to reverse the pattern of behavior of leucocytes derived from infected animals.

the inhibiting action of virulent cells of certain microorganisms, the injection of normal guinea pigs with homologous whole blood affected the leucocytes of the animal so that they became insusceptible to the test organism. However, the injection of blood taken from animals on the 4th day of intensive cortisone treatment (25 mg per day) into normal guinea pigs also induced the "insusceptibility" reaction, despite the fact that the blood was derived from animals whose peripheral leucocytes were susceptible to the inhibiting action of the test organism. In these two cases, therefore, it seemed that the mere injection of blood into the animals was sufficient stimulus to induce insusceptibility on the part of the recipient animal's leucocytes, irrespective of the pattern of behavior of the donor animal's leucocytes.

The leucocytes of normal animals were also influenced by cortisone although smaller amounts of the drug were effective in this case. Guinea pigs injected for 3 days with 1.3 mg cortisone per day were bled on the 3rd day. The leucocytes of such animals were insusceptible to virulent brucellae in vitro. However, 3 additional days of cortisone treatment, bringing the dosage to 7.8 mg, resulted in the appearance of leucocytes which displayed the normal susceptibility to the organisms, suggesting a refractory state to the drug and a reversal of the leucocyte behavior.

In view of the ease with which the leucocytic pattern could be altered, the microbial specificity of the reaction was studied. Animals were infected with virulent *Br. suis* and bled after 24 to 48 hours. The migration of leucocytes from these animals was unaffected by the presence in vitro of either the homologous organism or of the virulent H-37 Rv strain of *Myco. tuberculosis*, both of which organisms inhibited the migration of leucocytes of uninfected animals. On the other hand, leucocytes of normal animals were insusceptible to the presence of the C-203 strain of hemolytic streptococcus, while the same organism was able to inhibit effectively

the migration of leucocytes from animals infected with *Br. suis*. This inability of an organism to inhibit leucocytes of normal animals coupled with its ability to inhibit leucocytes normally insusceptible to the homologous infecting strain is a reaction for which we have at the moment no satisfactory explanatory data.

In vitro insusceptibility of leucocytes to virulent brucellae was also induced by injecting the animals with suspensions of dead brucellae cells, "native antigen" of *Br. suis*, and formalin subcutaneously.

Although the leucocytic migration was affected almost immediately upon either infection or treatment of the test animal by various substances, the insusceptibility of the leucocytes to virulent cells disappeared 3 months after bacterial infection had been established. At this time, despite residual infection in the animal, the in vitro leucocytic migration was inhibited by homologous virulent *Br. suis*.

DISCUSSION

The typical response of leucocytes of normal animals to the presence in vitro of microorganisms and their products as already described by others has been observed upon using brucellae. The genus *Brucella*, therefore, appears to possess the ability shared by certain groups of bacteria to inhibit directed leucocytic migration.

The phenomenon of leucocytic migration has been used as a tool in this study to examine the immediate consequences of infection. The use of the pretreated animal indicated that leucocytic migration per se was a most sensitive index of what might be considered part of the "general alarm syndrome" of Selye, for many of the substances known to induce this syndrome affected the susceptibility of leucocytic migration to certain microorganisms.

Although a variety of substances could be used to inject animals, thereby altering the susceptibility of their leucocytic migration to virulent bacteria, additional evidence of the general nature of the response was observed in the speed with which the inherent pattern of the response was altered, namely, at least one hour after treatment of the animal.

Thus, the limits of microbial specificity of the reaction, the speed with which leucocytic changes are induced, the inherent behavior of the leucocyte and its apparent independence of plasma substances, and finally the diverse nature of substances which have the ability upon injection into an animal of inducing the change in susceptility of its leucocytes with respect to migration, all suggest that the directed migration of leucocytes may be used to reflect the "general alarm reaction."

Since the fate of an infection may well be determined by the events occurring within three hours of its occurrence (Miles and Niven, 1950), as far as defense reactions are concerned the importance of the sensitivity of leucocyte migration to alteration becomes of considerable interest. We may see unfolding in a study of this phenomenon certain of the earliest phases of natural resistance wherein the leucocyte is able to be induced to overcome the inhibiting substances possessed by some virulent microorganisms. Any capabilities on the part of the leucocyte to affect the first hours of bacterial invasion may well determine the ultimate outcome of certain bacterial infections.

Further studies are now in progress both on the nature of the changes induced in the leucocyte, on the directed migration of nongranulocytic cells, and

on the possible role of this phenomenon as a factor influencing the ultimate fate of an infection, in terms proposed by the elegant experiments of Miles and Niven (1950).

SUMMARY

1. Virulent cells of brucellae inhibit directed migration of leucocytes of normal animals.

2. Avirulent brucellae failed to influence the migration of leucocytes of normal animals.

3. Leucocytes derived from infected animals are insusceptible to the inhibiting action of virulent brucellae upon their migration as early as 1 hour after infection.

4. Insusceptibility of leucocytes from pretreated animals is relatively nonspecific and may be induced by a number of widely different reagents.

5. It is suggested that the response of leucocytes from infected animals to virulent brucellae and mycobacteria is a part of the general adaptation syndrome or stress reaction and forms part of the immediate reaction to injury.

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REDUCTION OF INORGANIC SUBSTANCES BY YEASTS

I. EXTRACELLULAR REDUCTION OF SULFITE BY SPECIES OF *CANDIDA*

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In the course of studies on the mechanism of cellular division in yeasts, our attention was drawn to the utilization of reducing substances formed by the cells during glucose metabolism.[1,2] We have previously reported the presence in baker's yeast and in *Candida albicans* of systems utilizing reduced coenzyme II as hydrogen donor for the reduction of oxidized glutathione by glutathione reductase,[3,4] and transferring hydrogen from reduced coenzyme I to cystine in the presence of its reductase.[5] This paper describes sulfite reduction by yeasts with special reference to the pronounced abilities of some species of *Candida* to reduce inorganic sulfite when it is supplied as a bismuth complex in appropriate mediums. Certain species of *Candida* can reduce sulfite under these conditions to such an extent that the luxuriant cellular growth is jet black. With some species of *Candida* an extracellular reduction of sulfite has been observed. The phenomenon of extracellular reduction of sulfite has not been previously reported for a yeast, and is shown to result from the secretion of material which diffuses through agar but which cannot pass through a cellophane barrier.

The property of sulfite reduction has also been found to be useful for the recognition of certain species of *Candida*. A bismuth-sulfite medium appears to offer advantages for the initial isolation of *Candida* from clinical material.

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3. Conn, E. E. and Vennesland, B. 1951, Glutathione reductase of wheat germ. J. Biol. Chem. **192**: 17–28.
4. Mapson, L. W. and Goddard, D. R. 1951, The reduction of glutathione by plant tissues. Biochem. J. **49**: 592–601.
5. Nickerson, W. J. and Romano, A. H. 1952, Enzymatic reduction of cystine by coenzyme I (DPNH). Science, **115**: 676–678.

MATERIALS AND METHODS

The yeasts studied were maintained on malt agar slants and transferred at frequent intervals. Strains of yeasts were obtained from culture collections, or were isolated and identified in this laboratory. We particularly wish to thank Dr. Rhoda W. Benham and Dr. Juan E. Mackinnon for supplying isolates of *Candida*. Routine diagnostic mediums, commonly used in bacteriological laboratories, were employed in preliminary tests for sulfide formation by yeasts. Subsequent studies were conducted with mediums of known composition which have been designated as follows:

Acid bismuth yeast medium (*ABY medium*).—Glucose, 20.0 g; bismuth ammonium citrate, 5.0 g; Na_2SO_3, 3.0 g; $(NH_4)_2SO_4$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2$, 0.25 g; biotin, 10 μg; niacin, 500 μg; calcium pantothenate, 500 μg; Bacto-agar, 20 g; and distilled water to make 1 liter (final pH, 4.5).

Basic bismuth yeast medium (*BBY medium*).—Glucose, 20.0 g; bismuth ammonium citrate, 5.0 g; Na_2SO_3, 3.0 g; K_2HPO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2$, 0.25 g; biotin, 10.0 μg; Bacto-agar,

20 g; and distilled water to make 1 liter (final pH, 7.5).

Bismuth-GGY medium (*Bi-GGY medium*).—Glucose, 10.0 g; glycine, 10.0 g; yeast extract, 1.0 g; bismuth ammonium citrate, 5.0 g; Na_2SO_3, 3.0 g; Bacto-agar, 20 g; and distilled water to make 1 liter (final pH, 6.8). The Bi-GGY medium has also been used in many modifications which are indicated in pertinent sections of the text.

These mediums were not autoclaved. They were heated to about 80 C to disperse the agar and to cause a reaction between bismuth ammonium citrate and sodium sulfite. The newly formed "bismuth-sulfite" tends to settle out, and the mediums must be agitated while plates are being poured. Contamination of the mediums occurs very rarely. Freshly prepared plates of medium were employed. Characteristic patterns of growth were not obtained using culture medium that had been stored in the refrigerator for more than a few hours; only plate culture methods were used in the work described. These mediums were not found to permit characteristic patterns of sulfite reduction when distributed in test tubes for slant cultures.

The composition of the bismuth-sulfite which is formed in the Wilson-Blair bismuth sulfite medium,[6] long used by bacteriologists for the isolation of enteric pathogens, is as yet unknown. In all probability,* bismuth ammonium citrate and sodium sulfite interact to form a type of inorganic bismuth-sulfite. However, no attempts appear to have been made to prepare a bismuth-sulfite directly and to incorporate it into the medium. It was essential for our purposes to learn whether the yeasts reducing sulfite in the ABY, BBY, and Bi-GGY mediums could reduce bismuthyl hydroxy sulfites of known composition. Therefore, the complex bismuthyl-hydroxy-polysulfites described in Mellor[7] were prepared from sodium sulfite and bismuth nitrate according to the directions of Röhrig[8] and of Seubert and Elten.[9] Each of the complexes was incorporated in the glucose-glycine-yeast extract (GGY) agar (described under Bi-GGY), replacing the bismuth ammonium citrate and sodium sulfite. The complexes proved to be far from equal in the ease with which they were reduced by yeasts. Mediums prepared from the synthetic bismuth-sulfites were unsatisfactory if stored in the cold for more than a few hours, but prepared medium that had been stored in closed vials at room temperature for an extended period of time was found to support good growth and characteristic sulfite reduction.

RESULTS

Exploratory experiments were made with 3 different mediums frequently employed in testing for bacterial production of hydrogen sulfide. Bacto-bismuth sulfite agar (Wilson-Blair medium), Kligler's iron agar (Difco), and lead acetate agar (Difco) were inoculated from cell suspensions; the cultures were incubated at 20 C. No darkening of the growth or of the medium was observed with any yeast tested on Kligler's iron agar. This medium contains organic sulfur in peptones, yeast extract, and beef extract, and inorganic sulfur as sodium thiosulfate and ferrous sulfate. Sodium sulfite was added to this medium (1 g Na_2SO_3 per 500 ml medium) in one experiment, but there was no indication of sulfide formation with any yeast tested, even with yeasts known to reduce sulfite vigorously on

6. Wilson, W. J. and Blair, E. M. M. 1927, Use of a glucose bismuth sulphite iron medium for the isolation of *B. typhosus* and *B. proteus*. J. Hyg. **26**: 374–391; 1931, Further experience of the bismuth sulphite media in the isolation of *Bacillus typhosus* and *B. paratyphosus B* from faeces, sewage and water. J. Hyg. **31**: 138–161.

* The state of the sulfite in any of the so-called "bismuth-sulfite" mediums cannot be foretold from present knowledge of the chemistry of sulfur. Mellor lists 6 bismuth-sulfite complexes which have been prepared; these are all bismuthyl hydroxy polysulfite compounds varying in their degree of hydration and polymerization. The absence of simple compounds of bismuth and sulfur (except for bismuth trisulfide and bismuth sulfate) is remarkable; reported efforts to prepare the simple sulfite, thiosulfate, and dithionate of bismuth have not been successful.

7. Mellor, J. W. 1930, A Comprehensive Treatise on Inorganic and Theoretical Chemistry, Vol. X, London, Longmans, Green and Co.

8. Röhrig, A. 1888, Revision einiger älterer Angabe über schwefligsaure Salze. J. prakt. chem. **37**: 217–253.

9. Seubert, K. and Elten, M. 1893, Zur Kenntnis der basischen Metallsulfite. Ztschr. anorg. Chem. **4**: 44–95.

other mediums. On lead acetate agar a few yeasts exhibited a partial darkening of their growth. This medium contains organic sulfur in peptones and inorganic sulfur as sodium thiosulfate. The Wilson-Blair medium permitted irregular development of spotted dark growths of several species of yeasts; with 2 yeasts, a diffused darkening of the medium was observed. This medium introduces organic sulfur in peptone and beef extract and inorganic sulfur as ferrous sulfate and a bismuth-sulfite of undetermined composition. Though these preliminary tests indicated the ability of some species of yeasts to form sulfide in the presence of a suitable heavy metal (iron is evidently not suitable in view of the results with Kligler's medium), it was clearly not possible to study the reduction processes in complex mediums. The phenomenon of sulfite reduction was selected for further study. With mediums of known composition, a limited survey was made of the distribution of sulfite-reducing ability among yeasts. For certain species of *Candida*, with pronounced ability to reduce sulfite, the effect of various environmental factors on sulfite reduction has been examined.

Preliminary survey of the distribution of sulfite-reducing ability among yeasts.—Throughout this paper the term "yeast" is used in a broad sense; the genera *Candida*, *Brettanomyces*, *Geotrichum*, and *Trichosporon*, distinguished by some authorities as "yeast-like fungi", are considered, together with the Saccharomycetaceae and Cryptococcaceae, in the term "yeast." A comprehensive survey of the distribution of sulfite-reducing ability among yeasts has not been attempted. However, species representing 12 genera of yeasts have been examined (tables 1, 2, and 5).

By the expression "sulfite reduction" is meant the formation of a quantity of

TABLE 1.—*Appearance of different species of yeasts on ABY or Bi-GGY mediums after 48 hours of incubation at 28 C.*

Jet black:
- *Candida albicans*
- *Candida krusei* (rapidly becomes grey)
- *Candida tropicalis*

Dark brown:
- *Candida guilliermondi*

Brown or reddish brown:
- *Candida mortifera* (grey brown)
- *Candida parakrusei*
- *Candida pseudotropicalis*
- *Candida stellatoidea* (dark brown at 72 hr.)
- *Saccharomyces cerevisiae*
- *Torulopsis lactosa*
- *Torulopsis pulcherrima*
- *Zygosaccharomyces marxianus*
- *Zygosaccharomyces* sp. (strain no. 216)

Light brown or yellow brown:
- *Brettanomyces bruxellensis* (no growth on ABY)
- *Brettanomyces clausseni* (no growth on ABY)
- *Candida lipolytica*
- *Debaromyces klöckeri*
- *Sporobolomyces salmonicolor* (no growth on ABY)
- *Zygosaccharomyces priorianus*
- *Hansenula anomala* (brown within 120 hours)
- *Zygosaccharomyces sp.* (strain no. 290)

Colorless growth:
- *Candida pelliculosa*
- *Cryptococcus neoformans* (no growth on ABY)
- *Debaryomyces matruchotii*
- *Debaryomyces tyrocola*
- *Geotrichum lactis*
- *Kloeckera brevis*
- *Rhodotorula glutinis* (normal red color only)
- *Saccharomyces blanchardi* (no growth on ABY)
- *Saccharomyces lactis* (no growth on ABY)
- *Torulopsis acetiana*
- *Zygosaccharomyces acidifaciens*
- *Zygosaccharomyces bisporus*
- *Zygosaccharomyces lactis*
- *Zygosaccharomyces japonicus*
- *Zygosaccharomyces variabilis*
- *Zygosaccharomyces versicolor*

TABLE 2.—*Appearance of different species of yeasts on the BBY medium after 72 hours of incubation at 28 C.*

Jet black:
- *Candida albicans*
- *Candida guilliermondi*
- *Candida tropicalis*
- *Torulopsis pulcherrima*
- *Zygosaccharomyces priorianus*
- *Zygosaccharomyces* sp. (strain no. 223)
- *Zygosaccharomyces* sp. (strain no. 290)

Dark brown: (all turn black within 120 hours)
- *Candida parakrusei*
- *Candida pelliculosa*
- *Geotrichum lactis* (turns bluish black)
- *Hansenula anomala*

Brown or reddish brown:
- *Candida stellatoidea* (black within 120 hours)
- *Debaryomyces klöckeri*
- *Debaryomyces matruchotii*
- *Debaryomyces tyrocola*
- *Saccharomyces cerevisiae*
- *Zygosaccharomyces acidifaciens*

Light brown or yellow brown:
- *Candida krusei* (turns grey brown, then white)
- *Saccharomyces lactis*
- *Zygosaccharomyces* sp. (strain no. 216)

Colorless growth, or no growth:
- *Candida lipolytica*
- *Candida mortifera* (no growth)
- *Rhodotorula glutinis* (normal red color only)
- *Saccharomyces blanchardi*
- *Torulopsis lactosa*
- *Zygosaccharomyces bisporus*
- *Zygosaccharomyces lactis*
- *Zygosaccharomyces marxianus*
- *Cryptococcus neoformans* (no growth)

sulfide sufficient to combine with the heavy metal precipitant present in the medium so as to impart a perceptible darkening to the growth or to the medium. So far as is known, all yeasts are able to grow with sulfate as their sole source of sulfur, and many are known to grow with sulfite as their sole source of sulfur. Obviously, these sulfur sources must be reduced by the organism to form the organic sulfur compounds which are essential for the growth of yeasts. It is not known if yeasts forming appreciable quantities of sulfide, from the reduction of sulfite, do so by a route similar to that employed in the transformation of inorganic sulfur compounds during their biosynthesis of organic sulfur compounds.

Plates of ABY or Bi-GGY medium were inoculated heavily by streaking with cells from 48-hour slant cultures of a number of different yeasts. The plates were incubated at 28 C and examined daily for evidence of sulfite reduction. The extent to which the different organisms reduced sulfite in these mediums is described in table 1. Of the yeasts tested thus far, only 3 species, *Candida albicans*, *Candida krusei*, and *Candida tropicalis*, have been found to develop jet black growths on either of these mediums. The development of a dark brown color has, likewise, been confined to species of *Candida*. Within 48 hours the growth of *Candida guilliermondi* appeared dark brown, and *Candida stellatoidea* developed this intensity after 72 hours incubation. Over 40 different strains of *C. albicans* have been tested on the ABY or Bi-GGY mediums. Some of these strains had long been maintained in culture collections; others were freshly isolated from clinical material. All produced luxuriant jet black growths when the mediums were inoculated heavily with cells from young slant cultures. Five strains of *C. tropicalis*, including 3 recent isolates from clinical material, and 3 strains of *C. krusei* all gave characteristic growth patterns on these mediums. The appearance of plate cultures of different species of *Candida* when streaked on the ABY medium is shown in figure 1 (compare appearance of *C. albicans* on the Bi-GGY medium in fig. 3A; note absence of diffused blackening on this medium).

The Bi-GGY medium appears to offer promise for use in the isolation of *Candida* from clinical material. From such material isolated yeast colonies are generally obtained, bacterial growth being suppressed by the medium. Species of *Candida* may be readily distinguished on this medium, and the appearance of typical isolated colonies of *Candida* on the Bi-GGY medium is as follows:

C. albicans.—Circular, medium size colonies, frequently hemispherical, jet black, slight mycelial fringe, no diffusion; colony smooth on top, no prominence.

C. krusei.—Large colonies, flattened, wrinkled; a silvery black crown grading off to a brown periphery and extensive yellow halo; no diffusion.

C. parakrusei.—Colonies medium size, flattened, frequently wrinkled; glistening dark reddish brown, grading to light reddish brown periphery and extensive yellowish mycelial fringe.

C. pseudotropicalis.—Colonies rather large, flat, glistening dark reddish brown, slight mycelial fringe.

C. stellatoidea.—Medium size colonies, very dark brown, flattened, almost no mycelial development.

C. tropicalis.—Discrete colonies, medium size, dark brown with center black prominence; slight mycelial

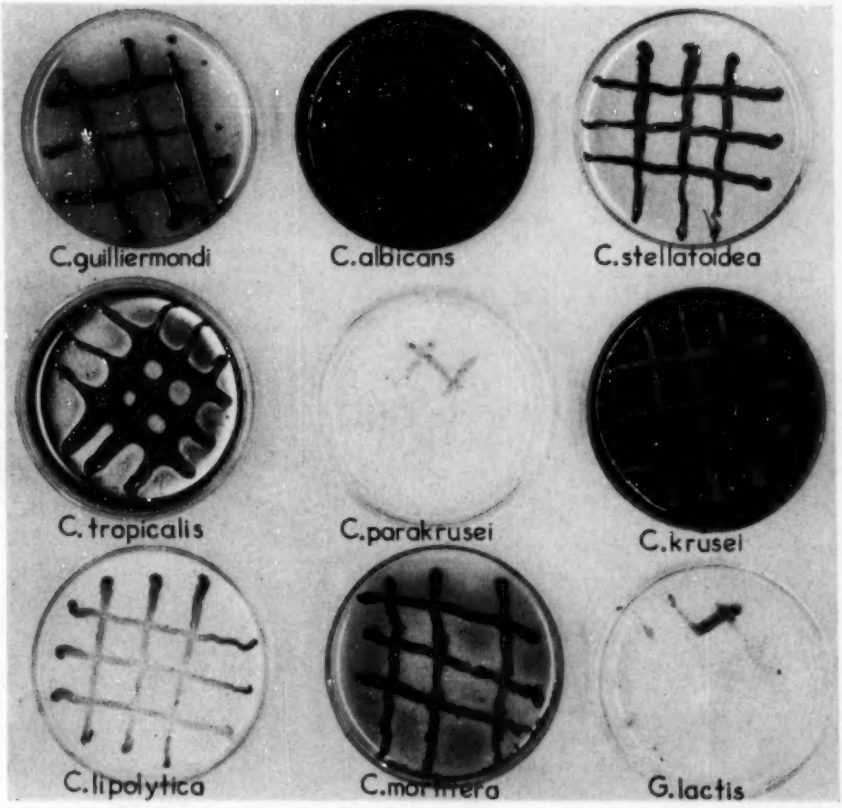


FIG. 1.—Growth of different species of *Candida* and of *Geotrichum lactis* on acid bismuth yeast medium (ABY). Cultures incubated 8 days. Blackening of cells and diffusion of sulfite-reducing material pronounced with *C. albicans* and *C. tropicalis; C. krusei* develops blackened cells that quickly become gray accompanied by a pronounced diffusion of the enzymatic sulfite-reduction system. The metallic sheen, developed on plates exhibiting diffusion, is well illuminated on the *C. tropicalis* plate.

fringe; diffused blackening frequently seen, with this species only, on the Bi-GGY medium.

The difference between the ABY and the BBY medium is mainly one of pH. Many more yeasts appeared dark brown or black on the BBY (pH 7.5) medium than on the ABY medium (pH 4.5) (see table 5). Table 2 lists the intensity of sulfite reduction, in terms of coloration of growth, for species of yeasts which have been tested on the BBY medium. Figure 2 shows the appearance of plate cultures of *Candida* on this medium.

Reduction of synthetic bismuthyl hydroxy polysulfite in GGY medium.—All of the complex bismuth-sulfites described in the literature were prepared and incorporated into the GGY medium. In addition, some previously undescribed complex bismuth-sulfites were prepared and tested. Most of this work will be described in another communication, but certain aspects bearing on the

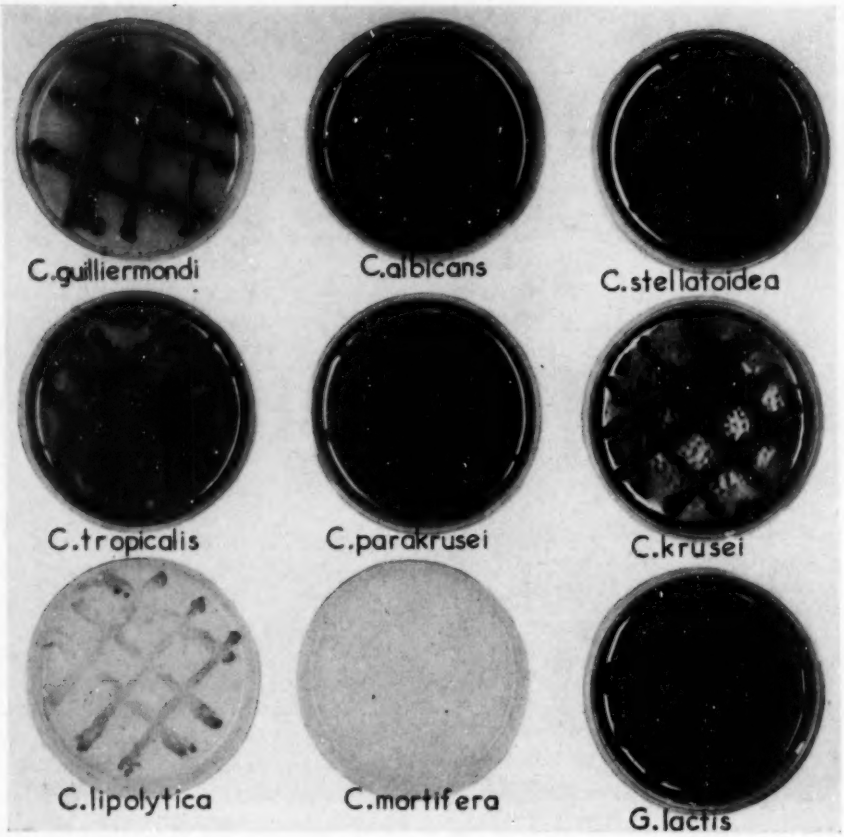
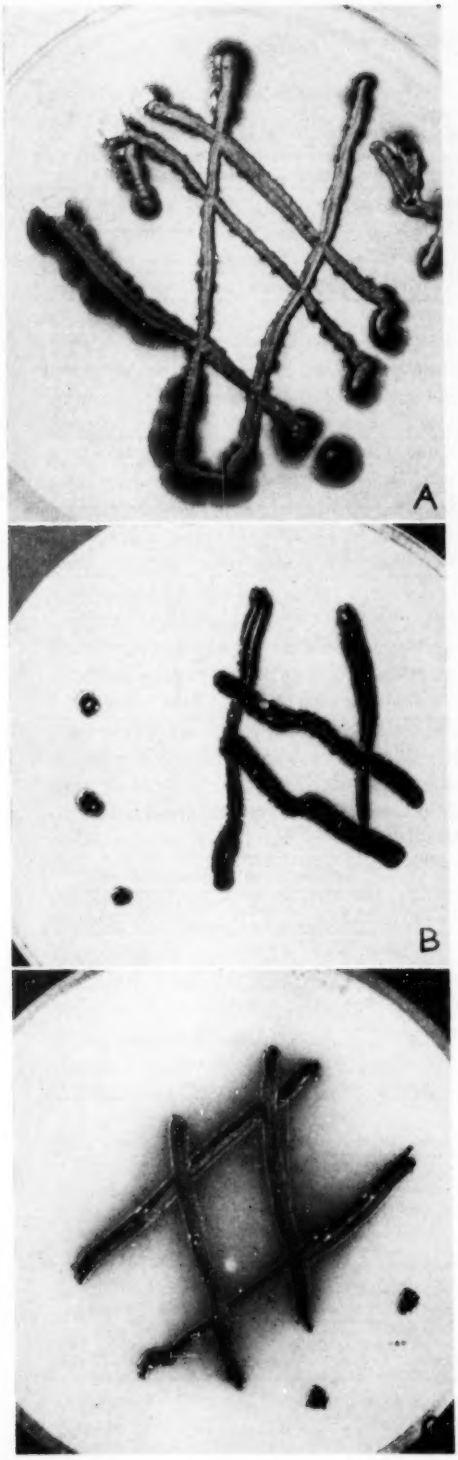


FIG. 2.—Growth of different species of *Candida* and of *Geotrichum lactis* on alkaline bismuth yeast medium (BBY). Cultures incubated 8 days. Note many more cultures exhibit diffused blackening than on ABY medium.

problem of sulfite reduction by *Candida* will be presented. As shown in figure 3B and C, species of *Candida* reduced a synthetic bismuthyl hydroxy polysulfite as markedly as they do the bismuth-sulfite formed by heating together bismuth ammonium citrate and sodium sulfite in the Bi-GGY mediums. In the Bi-GGY medium there is no diffused blackening of the medium with *C. albicans* (fig. 3A) and, similarly, there is none with a synthetic bismuth-sulfite,

FIG. 3.—*A*, culture of *C. albicans* on Bi-GGY medium incubated 14 days. Note extensive filamentation, black color of filamentous cells, and absence of diffused blackening of medium. *B* and *C*, medium prepared by adding 0.5 g of a synthetic bismuthyl-hydroxy-pentasulfite to 100 ml of GGY basal medium (replacing the bismuth ammonium citrate and sodium sulfite in Bi-GGY medium). *C. albicans* in *B*, and *C. tropicalis* in *C*. Note blackening of cells, filamentation, and absence of diffused blackening in *B*, and presence of diffused blackening in *C*. Distribution of diffused blackening in *C* follows inverse of general pattern of the so-called Langeron distribution of filamentation in *Candida* (compare with *A*).



as shown in figure 3B. *C. tropicalis* alone has been found to produce a diffused blackening of the Bi-GGY medium, and this is also obtained with a synthetic sulfite (fig. 3C). Certain of the synthetic bismuth-sulfites incorporated into the GGY basal medium support patterns of growth and sulfite reduction by *Candida* as described previously for the Bi-GGY medium. With a few of the synthetic substances the species differentiation can be obtained in slant cultures wherein the Bi-GGY mediums are unreliable owing to fading of the blackened growth of *C. albicans*.

The results shown in figures 3B and C tend to clarify the nature of the bismuth-sulfite present in the Bi-GGY, ABY and BBY mediums. There can be little doubt but that a form of bismuthyl hydroxy polysulfite is present in these yeast mediums.

Normal and mutant strains of C. albicans.—A large number of different strains of yeast, identified as *C. albicans* by microscopic observation of chlamydospore development, blastospore arrangement, and fermentation characteristics, have been tested on the three bismuth-sulfite mediums. In all cases the pattern of development thereon was closely similar and characteristic for the particular medium. From cultures of typical strains of *C. albicans* one may occasionally isolate morphological mutants which can be characterized as rough, permanently filamentous, or other growth type. Mackinnon[10] has described a number of types of permanently altered strains of *C. albicans* which can be classed as spontaneous morphological mutants. Three of these mutant forms were obtained from the culture collection of the Instituto de Higiene de Montevideo, through the courtesy of Dr. Juan E. Mackinnon, and their

10. Mackinnon, J. E. 1940, Dissociation in *Candida albicans*. J. Infect. Dis. **66**: 59–77.

TABLE 3.—*Reduction of sulfite by normal and by mutant strains of* C. albicans.

	Acid bismuth yeast agar (ABY)				Alkaline bismuth yeast agar (BBY)			
	Growth	Color	Sheen	Diffused blackening	Growth	Color	Sheen	Diffused blackening
C. albicans 1								
36 hr.	+++	Dark brown	0	0	+++	Black	+	+
60 hr.	+++	Black	++	+++	+++	Black	++	+++
108 hr.	+++	Black	++	++++	+++	Black	++	+++
C. albicans 35.1.1								
36 hr.	++	Dark brown	0	0	+++	Black	+++	++
60 hr.	+++	Black	+++	+++	+++	Black	+++	+++
108 hr.	+++	Black	++	+++	+++	Black	++	+++
C. albicans 75								
36 hr.	++	Dark brown	0	0	++	Dark brown	+	+++
60 hr.	++	Dark brown	0	+	+++	Black	+++	+++
108 hr.	++	Black	+	+++	+++	Black	++	+++
C. albicans IHM 582								
36 hr.	++	Brown	0	0	+++	Black	+++	++
60 hr.	+++	Dark brown	+	+	+++	Black	+++	+++
108 hr.	+++	Black	++	+++	+++	Black	++	+++
C. albicans IHM 805								
36 hr.	0	White	0	0	+	White	0	0
60 hr.	±	White	0	0	++	Light brown	++	++
108 hr.	±	Light brown	0	0	+++	Light brown	++	+++
C. albicans IHM 806								
36 hr.	±	White	0	0	+	White	0	0
60 hr.	+	Brown	0	0	++	Brown	++	++
108 hr.	++	Dark brown	+	+++	+++	Brown	++	+++
C. albicans IHM 807								
36 hr.	±	White	0	0	±	White	0	0
60 hr.	+	Light brown	0	0	±	White	0	0
108 hr.	++	Black	0	+	++	White	0	0

TABLE 4.—*Growth of eight species of* Candida *on mediums of known composition containing bismuth-sulfite.*

	Acid bismuth yeast medium (ABY)				Alkaline bismuth yeast medium (BBY)			
	Growth	Color	Sheen	Diffused blackening	Growth	Color	Sheen	Diffused blackening
C. albicans								
3 days	+++	Black	+++	+++	+++	Black	+++	+++
5 days	+++	Black	++	++++	+++	Black	++	+++
C. guilliermondi								
3 days	+++	Brown	0	0	+++	Black	++	±
5 days	+++	Black	0	±	++++	Black	+	±
C. krusei								
3 days	+	Grey-black	0	0	+++	Grey	+++	++*
5 days	+++	Grey	+	++++	+++	Grey	0	+++
C. lipolytica								
3 days	+	Light brown	0	0	+	White	0	0
5 days	++	Light brown	0	0	++	White	0	0
C. mortifera								
3 days	++	Light brown	±	±	0			
5 days	++	Brown	0	±	0			
C. parakrusei								
3 days	+	Light brown	0	0	++	Brown	+	0
5 days	++	White	0	0	+++	Dark brown	+	+
C. stellatoidea								
3 days	+	Brown	0	0	+++	Brown	+++	++
5 days	+++	Brown	0	±	+++	Black	++	++
C. tropicalis								
3 days	+++	Black	++	++	+++	Black	+++	++*
5 days	+++	Grey-black	++	++	+++	Grey-black	++	+++

* Black precipitate at rim of plate.

growth pattern was studied on the ABY and BBY mediums (table 3). Strain number IHM 582 is the parent strain, a typical strain of *C. albicans;* strains IHM 805 and 806 are filamentous under conditions which permit growth of the parent strain exclusively as a budding yeast; strains 805 and 807 are slow growing, and the latter has a low viability. All three of these mutants, in contrast to the parent strain, are nonpathogenic for experimental animals. The filamentous mutant 806 is only slightly slower growing than the normal strains and rapidly produces a black growth and considerable diffused blackening of the mediums. By its filamentous appearance it is readily distinguished from any normal strain of *C. albicans*.

Differentiation of C. stellatoidea *and* C. albicans *on ABY medium.*—An inspection of table 4 and comparison of figures 1 and 2 will reveal that *C. stellatoidea* is quite distinct from *C. albicans* in its pattern of growth on the two bismuth-sulfite mediums. Heretofore, the appearance of colonies of *C. stellatoidea* on blood agar plates has been the principal characteristic distinguishing this species from *C. albicans*, and it was on this basis that Jones and Martin[11] named the species. Diddens and Lodder[12] considered it to be merely a variety of *C. albicans*. The appearance of *C. stellatoidea* on the ABY medium shows it to be different from *C. albicans*. Interestingly enough, Jones and Martin found *C. tropicalis* and *C. albicans* to be antigenically identical, whereas *C. albicans* and *C. stellatoidea* were antigenically different.

Use of bismuth-sulfite mediums for isolation and identification of Candida.—The suggested usefulness of the Bi-GGY and ABY mediums for the isolation and identification of species of *Candida* has been examined by other workers in several laboratories. Through the courtesy of Dr. H. W. Schoenlein of the Difco Laboratories, experimental batches of these mediums were prepared and distributed. In commenting on his results with the Bi-GGY medium, Gordon* stated, "It has been found possible to separate all 8 of our species of *Candida* on the basis of their appearance on this medium. . . . The new mediums may prove to be of value in rapid isolation and identification of *C. albicans* from mixed cultures or contaminated materials and the Bi-GGY medium shows promise as a rapid means for identifying all *Candida* species. If this can be worked out it would certainly save time and trouble over the long method involving fermentations and other criteria." Fischer* also found that characteristic colonies were produced by the different species of *Candida* on the Bi-GGY medium. These colony types were readily picked out when the Bi-GGY medium was used for routine isolation from clinical material.

Development of a diffused blackening of bismuth-sulfite mediums.—An outstanding feature accompanying the growth of a few yeasts on mediums containing bismuth-sulfite is the development of

11. Jones, C. P. and Martin, D. S. 1938. Identification of yeast-like organisms isolated from the vaginal tracts of pregnant and non-pregnant women. Am. J. Obst. & Gynec. **35**: 98–106.

12. Diddens, H. A. and Lodder, J. 1942. Die Hefesammlung des "Centraalbureau voor Schimmelcultures" Beiträge zu einer Monographie der Hefearten. II Teil, Die anaskosporogenen Hefen. Zweite Hälfte, N. V. Noord-Hollansche Uitgevers Maatschappij Amsterdam.

* The author wishes to thank Dr. Morris A. Gordon, Mycology Laboratory, Communicable Disease Center, Chamblee, Georgia, and Dr. J. B. Fischer, Central Laboratory, Department of Health, Toronto, Ontario for their kindness in testing the mediums and supplying the above information.

TABLE 5.—*Number of species of different genera of yeasts producing a dark brown or black growth on bismuth-sulfite yeast mediums.*

Genus	ABY medium		BBY medium		Bi-GGY medium	
	Species tested	Number dark	Species tested	Number dark	Species tested	Number dark
Brettanomyces	2	0			2	0
Candida	9	3	9	6	9	3
Cryptococcus	1	0	1	0	1	0
Debaryomyces	3	0	3	0		
Geotrichum	1	0	1	0	1	0
Hansenula	1	0	1	1		
Kloeckera					1	
Pullularia					1	0
Saccharomyces	2	0	2	0	1	0
Sporobolomyces	1	0			1	0
Rhodotorula	1	0	1	0	1	0
Torulopsis	3	0	3	1		
Zygosaccharomyces	6	1	6	3	2	0

With many species, several different isolates have been tested.

a diffused blackening of the medium. The three mediums employed in this study differ in the number of yeasts producing blackening thereof. This is evident from a comparison of figures 1, 2 and 3. The following yeasts produced diffused blackening of the different mediums after 72 hours incubation:

1. Bi-GGY medium: *C. tropicalis.*

2. ABY medium: *C. albicans C. krusei* and *C. tropicalis.*

3. BBY medium: *C. albicans C. guilliermondi* (slight diffusion) *C. krusei, C. pelliculosa* (slight diffusion), *C. stellatoidea, C. tropicalis, Geotrichum lactis, Hansenula anomala* (slight diffusion), *Saccharomyces cervisiae* (slight diffusion) and *Zygosaccharomyces sp.* (strain no. 290).

After 120 hours' incubation a slight diffused blackening was produced on the BBY medium by cultures of *C. parakrusei* and *Zygosaccharomyces sp.* (strain No. 216).

Three conditions characteristically accompanied the diffused blackening of the bismuth sulfite mediums: (1) A pronounced metallic sheen appeared over the agar surface (except faintly with *C. tropicalis* on the Bi-GGY medium). (2) The diffused blackening occurred only on the surface of the agar; the black material consisted of a very thin film; if stripped off from the surface, it again developed as a thin black film within 4 to 5 hours. (3) The diffused blackening rapidly spread over the agar surface; the agar surface of a large Petri dish (15 cm diameter) was completely darkened within 72 hours by the metabolic activity of cells in a streak made near the center of the dish.

The diffused blackening produced by yeasts on the bismuth-sulfite mediums might result from any of several causes. Certain of these possible causes have been examined and evidence for or against each will be considered. Possible causes are: (1) the bismuth sulfide may diffuse from the cells of the blackened yeasts, (2) diffusion of some other sulfide (organic or inorganic) from the cells, (3) volatilization of hydrogen sulfide from yeast cells, (4) diffusion from the cells of some organic reducing substance which can reduce sulfite, and (5) diffused blackening may result from the action of enzymatic material which has been secreted by the cell and which catalyzes the reduction of sulfite by hydrogen donors that have been produced in the cell during the course of glucose metabolism and secreted into the medium.

To consider the possibilities just suggested: (1) Diffusion of bismuth sulfide from yeast cells—if powdered bismuth sulfide is deposited in a spot on the surface of a bismuth-sulfite agar, there is no spread of blackening. (2) Diffusion

of some other sulfide from cells appears to be improbable since addition of a strong solution of sodium sulfide to assay cups placed on the surface of a bismuth-sulfite agar caused the appearance of an intensely black zone of small diameter around the cup with intense blackening of the entire depth of the agar; the black zone surrounding the cup showed no metallic sheen. (3) Volatilization of hydrogen sulfide has not been detected in yeast cultures on a bismuth-sulfite agar which show diffused blackening; a piece of moistened lead acetate paper fastened to the under side of a Petri dish lid covering such culture did not blacken. Blackening of a bismuth-sulfite agar always progresses outward from the colony; the rate of progression has been estimated to be a few mm^2 per hour. The surface does not darken rapidly and evenly as it would were hydrogen sulfide volatilized. The possible explanations of diffused blackening of the mediums suggested as numbers 4 and 5 may be considered separately and in more detail.

Diffusion of reducing substances from yeast cells.—Two special plate culture techniques were employed to obtain further evidence on the nature of the diffused blackening and to learn whether the presence of either bismuth or sulfite in the growth medium was necessary for the elaboration of diffusible sulfite-reducing substances. One, which may be called an "annulus technique," consisted of forming an inner agar cylinder (GGY agar, or other growth medium, containing neither bismuth nor sulfite) surrounded by a concentric zone of ABY medium (fig. 4). In this technique, the agar medium was poured into small Petri dishes and rapidly solidified by floating the dishes in ice water. The agar disk was expelled with the aid of a sterile spatula into a sterile, empty Petri dish of larger size (100 mm diameter). If the thick GGY agar disk was fairly well centered in the larger dish, it proved to be easy to pour melted bismuth-sulfite agar (ABY) around the disk, establishing contact between the mediums, yet not allowing the bismuth-sulfite agar to reach the top of the GGY agar disk or to contaminate the top of it. As soon as the second agar had hardened, the inner disk of GGY agar was inoculated and plates were incubated at 28 C.

The second plate culture method may be called a "half-plate" method (fig. 4). In this instance sufficient bismuth-sulfite agar medium (ABY) was poured into standard petri dishes, which were inclined so as to cover only half the bottom area. When this agar was solid, the plates were set flat on the bench and a second pouring of agar was made (using GGY agar) so as to bring the new medium into contact with, but not overlapping more than 1 to 2 mm of the first agar. When the second agar had solidified, the GGY side of the plate was inoculated.

As will be seen from figure 5, *C.*

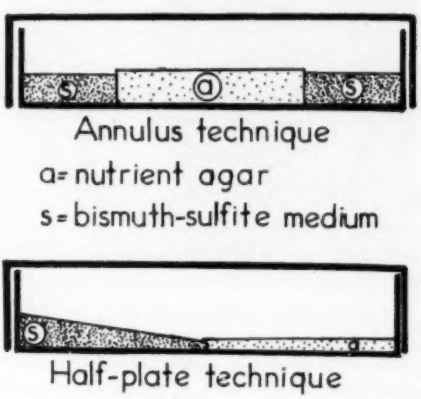


FIG. 4.—Diagram of annulus technique and of half-plate technique for demonstrating secretion and diffusion of materials from microorganisms and for studying nutritional requirements for production of diffusible substances.

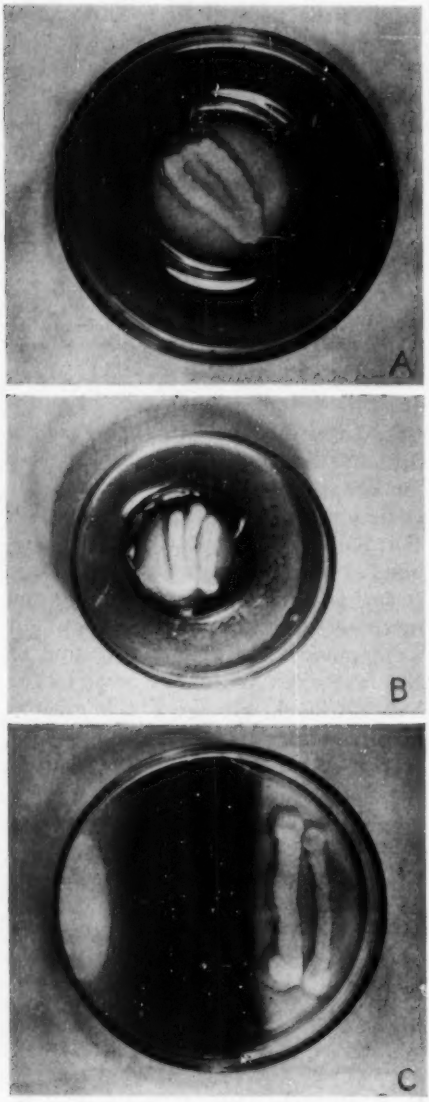


FIG. 5.—Demonstration of extracellular reduction of bismuth-sulfite by material diffusing from growing cells of *Candida*. In *A* and *B* are shown 4-day cultures of *C. krusei* on "annulus plates"; *C* shows a 4-day culture of *C. albicans* on a "half-plate." In all cases the organism is growing on plain nutrient medium and the (ABY) agar containing bismuth-sulfite is darkened only by material diffusing into it. In *A* center agar is acetate-glucose-yeast extract; in *B* and *C* the nutrient medium is GGY.

albicans and *C. krusei* produced zones of intense black coloration at the junction of the bismuth-sulfite medium with the GGY medium in plates prepared by both the "annulus" and "half-plate" methods. By the same techniques, plates inoculated with *Saccharomyces cerevisiae* showed no blackening at the junction of the mediums. Evidently, both species of *Candida* are able to produce reducing material which diffuses from the cells through some distance of an agar medium, and causes the reduction of bismuth-sulfite to bismuth sulfide on coming in contact with the bismuth-sulfite zone. The blackening shown in figure 5 always occurred at the zone of contact of the two mediums if freshly prepared plates were inoculated. The zone of blackening might extend several mm inside the GGY area if plates were aged 1 to 2 weeks before inoculation, indicating that the bismuth-sulfite had diffused some distance into the GGY agar in this storage time. No spontaneous darkening of the uninoculated plates, either at zone of contact of the two mediums, or elsewhere, has ever been observed even after prolonged storage of plates.

Enzymatic nature of sulfite reduction by yeasts.—The participation of an enzymatic reaction in the extracellular process of sulfite reduction has been demonstrated in the following manner. Although the materials responsible for sulfite reduction diffuse rapidly throughout an agar medium, diffusion of some component of the active material is completely prevented by a cellophane membrane (cellophane tubing, Dupont). Cellophane tubes of 2 cm diameter were sealed at one end, sterilized, and filled with ABY or GGY agar medium. These bags, with medium, were then placed in an upright position in a crystallizing dish and ABY medium was poured around them. After the outer medium had solidified, the medium inside the

bags was inoculated with *C. albicans*. Blackening of the cells and surface of the ABY medium inside the cellophane boundary progressed normally. When blackening of the ABY medium had spread as far as the boundary, it did not progress farther, even on prolonged incubation. Similarly, with GGY medium inside the cellophane, no diffused blackening of the ABY medium outside has been observed. Therefore, the extracellular sulfite reduction process is the result of the operation of a system that is released from cells under certain nutritional conditions, is capable of diffusion through agar for at least 10 cm, but contains a component which is retained on dialysis against 2% agar. Sulfite reduction has been obtained recently in cell-free preparations of *C. albicans*, and the properties of the system will be described in a subsequent communication.

DISCUSSION

The data demonstrate that the ability to reduce a bismuthyl hydroxy sulfite is common, in greater or less degree, to many yeasts. Of the yeasts examined, the most pronounced sulfite-reducing abilities were exhibited by species of the genus *Candida*.* The property of extracellular reduction of bismuth-sulfite to bismuth sulfide in acidic or neutral mediums is confined, thus far, to three species of yeasts, *C. albicans*, *C. krusei*, and *C. tropicalis*.

This is possibly the first demonstration of extracellular reduction of an inorganic sulfite by yeasts and of the participation of an enzymatic step in the extracellular reduction process. Although conversion of sulfite to sulfide must involve the participation of strongly reducing substances, the reduction achieved by the material diffusing from cells of *C. albicans* occurs only on the surface of the agar. Slants of ABY medium inoculated with *C. albicans* exhibit diffused blackening only on the surface of the slant. Stab cultures showed no diffused blackening in the depths of the medium.

From measurements on the rate of spread of the diffused blackening of ABY medium by *C. albicans*, it is believed that there is only one enzymatic component in the material responsible for the reduction of bismuth-sulfite. Some evidence is available to indicate that the enzymatic step is not directly responsible for the reduction of the complex sulfite. Rather, it appears that the enzymatic step causes the reduction of an organic disulfide constituent of the secreted material.† The organic sulhydryl substance thus obtained is then capable of reducing the complex bismuth-sulfite to give the surface blackening and sheen. The sulfhydryl material could not be glutathione since the addition of glutathione, as a powder or in solution, to the surface of ABY agar produced very little reduction, no sheen, and almost no diffusion. Cysteine, however, could be the substance responsible for reduction of bismuth-sulfite. When added to the surface of ABY medium it causes a blackening, accompanied by a metallic sheen, that is diffused over the surface but not into the depth of the agar. Investigations on the mechanism of sulfite reduction will be presented in another communication.

The highly reducing material secreted by *C. albicans* and *C. tropicalis* may play some role in the disease processes caused by these yeasts. The significance

* *C. albicans* and *C. tropicalis* have recently been found to reduce potassium tellurite (0.1 g per 100 ml of GGY medium) to black metallic tellurium. *S. cerevisiae* gave not the slightest trace of blackening of this medium. Further details will be reported in a subsequent paper.

† The reaction may involve the participation of cystine reductase described by Nickerson and Romano.[5]

of the phenomenon in the pathogenesis of moniliasis might well be examined.

SUMMARY

The ability of some fifty different yeasts, representing twelve genera, to reduce sulfite to sulfide has been examined with a variety of mediums and three heavy metal indicators. When present in mediums as a complex bismuthyl hydroxy compound, sulfite reduction was observed to occur to some extent with many of the yeasts tested. Pronounced reducing abilities, resulting in the development of jet black growths on certain bismuth-sulfite agar mediums, were exhibited only by species of *Candida*. The secretion of material accomplishing the extracellular reduction of sulfite under aerobic conditions in an acidic environment was demonstrated with three species of *Candida*, *C. albicans*, *C. krusei*, and *C. tropicalis*. The participation of an enzymatic reaction in the extracellular reduction of bismuthyl hydroxy sulfite has been demonstrated. Secretion of the sulfite-reducing system does not depend on the presence of sulfite or bismuth in the medium as demonstrated by two different plate culture techniques.

The usefulness of certain of the bismuth-sulfite yeast mediums for the isolation and identification of species of *Candida* has been described.

THE RELATIONSHIP OF THE ANTIGENIC CHARACTERISTICS OF STREPTOCOCCI AND SPECIFIC ANTIBODY RESPONSES FOLLOWING STREPTOCOCCAL INFECTIONS

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Extensive studies of infections due to group A β hemolytic streptococci have been made by clinical observation and serologic methods. The hemolytic streptococcus has been made the subject of numerous investigations relating to antigenic, enzymatic and physiologic characteristics, and considerable information has been accumulated which pertains to the clinical features of infection with β hemolytic streptococci and of subsequent antibody responses in patients. However, attempts to relate antigenic and enzymatic properties of the infecting organism to the clinical and antibody response of individual patients have been infrequent. Such a correlation of clinical and antibody response in β hemolytic streptococcal infections with some of the biological characteristics of the homologous organism producing these responses is presented in this study.

MATERIALS AND METHODS

More than 150 patients and 185 strains of streptococci were involved. Many of these were not included because of inadequacies in data, serum specimens, or cultures. There are 111 individuals and corresponding strains in the series. Fifty-one students in the outpatient department or infirmary of the Department of Preventive Medicine and Student Health at the University of Wisconsin and 60 recruits at the U. S. Naval Training Station, Great Lakes, Illinois were used. Students at the University of Wisconsin were selected for study on the basis of the following criteria:‡ (1) the history of sudden onset of a sore throat with dysphagia, (2) evidence of acute pharyngitis and/or tonsillitis determined by the presence of pharyngeal mucosal and tonsillar injection, swelling of these structures and exudate, (3) anterior superior cervical lymph node enlargement and tenderness, (4) fever of 100 F or more, (5) leucocytosis and (6) a throat culture showing β hemolytic streptococci. The 60 patients selected at Great Lakes were not consecutive cases, but were selected from a larger group of recruits treated in the infirmary for an exudative lesion on the tonsils or pharynx. Their throat cultures were all positive for β hemolytic streptococci. These men all had a temperature of 100 or over and were admitted to the infirmary for treatment. The cases were placed in treatment or control groups in strict rotation.

Five patients who developed rheumatic fever following their throat infection are included. Of these, 4 occurred in the recruits and were selected from a much larger group; this does not represent the incidence of rheumatic fever in the 111 cases. The 5th case of rheumatic fever occurred among the student group. Maxillary sinusitis developed in 4, otitis media in 2 and rheumatic fever in 5 patients. Each patient was observed throughout the acute infection and from 2 to 12 weeks thereafter. From the recruits a blood specimen was obtained within the first few days of arrival at Great Lakes, and again at the 5th week and 10th week of the training period. Further blood specimens were obtained at the time of onset of the streptococcal infection and at least

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‡ The necessity of obtaining complete clinical data, the infecting strain of streptococcus and blood specimens on each patient during the acute and convalescent phase made selection in strict rotation impossible.

once, usually more often, during the convalescence at periods not less than 2 weeks after the onset of symptoms. From the students, blood specimens were obtained at the time of onset of symptoms and during convalescence. The serum was separated from the clot within 24 hours. That obtained from students was stored at 4 C and that from recruits was frozen and kept at −20 C. Treatment of the naval recruits consisted of one of the following schedules: (1) on admission, an initial intramuscular injection of 300,000 units of procaine penicillin in aqueous suspension with 300,000 units of a soluble salt of penicillin G; subsequent doses consisted of 300,000 units of procaine penicillin at 12, 24 and 36 hours and a final injection at 48 hours of procaine penicillin in oil, with 2% aluminum monostearate, 600,000 units; (2) crystalline procaine penicillin G, in aqueous suspension, 600,000 units on admission and at 48 and 96 hours or at 72 hours only; the dosage for the patients in the Student Infirmary ranged from a single injection of 400,000 units of procaine penicillin to 7,800,000 units of procaine penicillin fortified with a soluble salt of penicillin G, in a period of 5 days; the treatment most often prescribed was 300,000 units of procaine penicillin fortified with 100,000 units of a soluble salt, intramuscular, twice daily for 4 days; all patients from each source became afebrile within 48 hours.

Throat cultures were prepared by the pour plate technique, using sheep blood agar. The strains of streptococci isolated were stored in vacuo, after drying in the frozen state. Lancefield group and type determinations* were done on each, using a method similar to that of Swift, Wilson and Lancefield.[1] Grouping serums for groups A, B, C, D and G and typing serums for 37 different types were employed. Strains which gave no type specific reaction were labeled "no type" (NT). Each strain was tested for production of the following substances by the method of indicated:

Streptokinase.—Eighteen-hour cultures in beef heart infusion broth containing 1% dextrose and 0.001% phenol red were tested by the fibrin clot lysis method of Kaplan et al.[2] During the last 4 to 6 hours of incubation the cultures were maintained at a pH of from 7 to 7.6 by the addition of 1*N* sodium hydroxide. Sixty-seven culture supernates in which clot lysis in a 1:2 dilution was absent after 30 minutes incubation were held for 18 hours. Fifty of these were thereby demonstrated to be streptokinase-positive. Controls were consistently negative.

Hyaluronic acid.—A semiquantitative method previously described[3] was employed for the determination of hyaluronic acid. This consists of layering 24-hour culture supernates in serial 2-fold dilutions from 1:10 to 1:80, over acidified normal horse serum. The development of a precipitate at the interface was considered to be a positive reaction.

Hyaluronidase.—Two methods were used for the detection of hyaluronidase. One was based on Pike's observations that certain strains of β hemolytic streptococci elaborate first hyaluronic acid, and after prolonged incubation begin to produce hyaluronidase. All of the cultures showing the presence of hyaluronic acid in 18 hours were therefore incubated for 7 days, during which time the pH was held at about 7.6. After this time tests for hyaluronic acid were again made. If none was demonstrable it was assumed that hyaluronidase had been elaborated in the aging culture. Tests by the mucin-clot-prevention technique on such 7-day culture supernates were usually but not always positive for hyaluronidase.

It should be pointed out that the technique described by Pike[4] for the demonstration of hyaluronic acid, namely, turbidity after mixing equal parts of undiluted culture supernate and acidified serum, is subject to error when cultures which have been frequently neutralized are used. It appears that the large amount of buffer produced by such neutralization prevents the formation of the hyaluronic acid-serum protein complex because of excess alkali. Dilution of the culture supernate as in the ring method, or acidification of the acid serum-culture supernate mixture to pH 4 results in precipitation. Of 146 strains, 16 were shown to form hyaluronic acid during the first 8 hours of growth followed by destruction of this polysaccharide after 7 days incubation. Ten additional strains would apparently have given similar results by Pike's technique for the deter-

* Grouping and typing serums were furnished through the courtesy of Dr. Elaine L. Updyke, Communicable Disease Center, Streptococcus Laboratory, Chamblee, Georgia.

1. Swift, H. F., Wilson, A. F. and Lancefield, R. C. 1943, Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exper. Med. **78**: 127–133.
2. Kaplan, M.H. 1946, Studies of the streptococcal fibrinolysis. III. A quantitative method for the estimation of serum antifibrinolysin. J. Clin. Invest. **25**: 346–351.
3. Seastone, C. V. 1943, The occurrence of mucoid polysaccharide in hemolytic streptococci of human origin. J. Exper. Med. **77**: 21–28.
4. Pike, R. M. 1948, Streptococcal hyaluronic acid and hyaluronidase. I. Hyaluronidase activity of noncapsulated group A streptococci. J. Infect. Dis. **83**: 1–11.

mination of hyaluronic acid; however, by the ring test on diluted supernate or by the acidification of the culture, hyaluronic acid was shown to be still present after 7 days.

The other method for the determination of hyaluronidase was based on the mucin-clot-prevention technique of McClean.[5] The details of the procedure have been previously described.[6] The usual incubation time of the mixture of enzyme and substrate is 20 minutes. When no enzyme was demonstrable after this period, another sample of the same supernate was incubated with substrate for 18 hours. In this manner enzyme sometimes could be demonstrated after 18 hours when none was evident after 20 minutes. In an effort to find a more sensitive test for hyaluronidase, heavily encapsulated group C streptococci were incubated in the supernate for an hour and examined for loss of capsular material. This method did not prove to be as sensitive in detecting the presence of enzyme as did the 18-hour mucin-clot-prevention test and was therefore abandoned.

Anti-streptolysin O.—The method for determination of anti-streptolysin O was that described by Todd[7] and modified by Hodge and Swift.[8] Serum dilutions were made in increments of 50. Serums with titers higher than 1:500 were further diluted in increments of 100. It was assumed that streptolysin O was produced by most of the strains examined, although this was not specifically determined.

Anti-streptokinase.—The method described by Kaplan et al[2] was employed for the determination of anti-streptokinase. Serum dilutions were made according to Kaplan's procedure.

Anti-hyaluronidase.—To determine the presence of anti-hyaluronidase in serum, the mucin-clot-prevention test was employed. Twofold dilutions of the patients' serums were incubated with a constant amount of group A streptococcal hyaluronidase, following which a substrate consisting of potassium hyaluronate, a 1:10 dilution of horse serum in physiological saline and water in a ratio of 1:1:2, was added and the mixture allowed to incubate for 20 minutes. On the addition of 0.2 *N* acetic acid the first tube in which a clot failed to appear was taken as the end point of the reaction. The serums of patients from whom group C strains were isolated were tested against hyaluronidase from a strain of group C streptococcus.

A two tube rise in titer for any one of the three antibodies has been interpreted arbitrarily as indicating a significant increase in antibody.

*Statistical analysis.**—The data were subjected to statistical analysis, and the terms significant or not significant are employed in a statistical sense. For purposes of comparison, the patients were divided into 4 treatment groups: (1) those who received more than 1,500,000 units of penicillin and in whom an effective blood level of penicillin could be expected for 4 days or more, (2) those who received less than 1,500,000 units of penicillin over a period of less than 2 days, (3) those who received aureomycin, terramycin or sulfonamides (3 patients only) and (4) those who received no antibiotics or chemotherapeutic agents.

RESULTS

In presenting the results, each of the four biological characteristics under investigation, namely, streptolysin, hyaluronidase, hyaluronic acid and streptokinase, has been considered separately in relation to the Lancefield group and type, the antibody response of the patient and the effect of antibiotic therapy on this antibody response. In table 1, which summarizes the response to streptolysin O, Lancefield group and type are not presented since it was assumed that all of the organisms in the study produced this toxin. It may be seen that antibiotic treatment appears to suppress to some extent the development of anti-streptolysin O, but the difference between the treated and nontreated groups is not significant. The observations of Kilbourne and Loge[9] and Anderson,

5. McClean, D. 1943, Studies in diffusing factors II. Methods of assay of hyaluronidase and. their correlation with skin diffusing activity. Biochem. J. **37**: 169–177.
6. Quinn, R. W. 1948, Studies of the mucin-clot prevention test for the determination of anti-hyaluronidase titre of human serum. J. Clin. Invest. **27**: 463–470.
7. Todd, E. W. 1932, Antigenic streptococcal hemolysins. J. Exper. Med. **55**: 267–280.
8. Hodge, B. E. and Swift, H. F. 1933, Varying hemolytic and constant combining capacity of streptolysins; influence on testing for anti-streptolysins. J. Exper. Med. **58**: 277–287.

* Statistical analyses were performed under the direction of Dr. Margaret P. Martin.

9. Kilbourne, E. D. and Loge, J. P. 1948, The comparative effect of continuous and intermittent penicillin therapy on the formation of antistreptolysin in hemolytic streptoccal pharyngitis. J. Clin. Invest. **27**: 418–424.

TABLE 1.—*Anti-streptolysin O response in treated and untreated patients.*

	Treatment A*		Treatment B*		Treatment C*	Untreated patients		Total patients	
	Number	Percent	Number	Percent	Number	Number	Percent	Number	Percent
Number of patients showing antibody rise	29	60.4	14	58.3	2	27	75.0	72	64.9
Number of patients showing no antibody rise	19	39.6	10	41.7	1	9	25.0	39	35.1
Total patients	48	100.0	24	100.0	3	36	100.0	111	100.0

* In this and succeeding tables, treatment A consisted of a *minimum* of 1,500,000 units of penicillin over a 4-day period. Many of the patients received more than this. Treatment B consisted of a *maximum* of 1,500,000 units of penicillin over 2 days or less. Treatment C was aureomycin, 2 g in 2 days, terramycin, 2 g in 2 days, or sulfadiazine, 2 g in 2 days.

TABLE 2.—*The effect of time of treatment on anti-streptolysin O, anti-streptokinase and anti-hyaluronidase responses.*

	Anti-streptolysin O				Anti-streptokinase				Anti-hyaluronidase			
	Antibody rise	No antibody rise	Total	Per cent rise	Antibody rise	No antibody rise	Total	Per cent rise	Antibody rise	No antibody rise	Total	Per cent rise
Patients treated less than 2 days after onset	24	12	36	66.7	18	18	36	50.0	17	19	36	47.2
Patients treated 2 or more days after onset	22	17	39	56.4	16	23	39	41.0	10	29	39	25.6
Patients receiving no treatment	26	10	36	72.2	17	19	36	47.2	15	21	36	41.7
Total	72	39	111	64.9	51	60	111	45.9	42	69	111	37.8

Kunkel and McCarthy[10] show a more striking effect on this antibody production that was observed in the present study. In table 2 it appears that the time elapsing between the onset of symptoms and the beginning of therapy has no marked effect on the development of specific antibodies.

The data related to hyaluronic acid and hyaluronidase are shown in tables 3 and 4. The majority (76%) of the strains produced hyaluronic acid, either alone or in addition to hyaluronidase. This enzyme, when it appeared in a hyaluronic acid-producing strain could usually be detected only after 7 days incubation and then only after an 18-hour contact with the substrate. The development of anti-hyaluronidase was seen in infections in which the enzyme could not be demonstrated in the infecting organism. Moreover, the antibody response to hyaluronidase was considerably less marked in infections with strains producing hyaluronidase alone; strains producing neither enzyme nor substrate failed entirely to stimulate

TABLE 3.—*Relationship of hyaluronic acid and hyaluronidase production and Lancefield group and type.*

G&T	Hyaluronic acid alone produced	Hyaluronidase alone produced	Hyaluronic acid and hyaluronidase produced	Neither hyaluronic acid nor hyaluronidase produced	Total
A 1	6		1	1	8
A 3	7	1	2		10
A 5	14		4	2	20
A 6					
A 12			1		1
A 14	10		6		16
A 18	2		1		3
A 19	1				1
A 30	1				1
A 39	2	1			3
A 42	1			1	2
A NT	12	7	4	2	25
NG	1	1	2		4
C		7			7
G		1			1
?	2	1			3
Total	63	19	22	7	111

In this and succeeding tables; G = Lancefiele group, T = Lancefield type, NT = strain could not be typed, NG = strain could not be grouped.

Figures above indicate the number of strains in each category.

10. Anderson, H. C., Kunkel, H. G. and McCarthy, M. 1948, Quantitative antistreptokinase studies in patients infected with group A hemolytic streptococci. J. Clin. Invest. **27**: 425–434.

TABLE 4.—*Relationship of hyaluronic acid-hyaluronidase production, and anti-hyaluronidase in treated and untreated patients.*

Anti-hyaluronidase response	Hyaluronic acid alone produced				Hyaluronidase alone produced				Hyaluronic acid and hyaluronidase				Neither hyaluronic acid nor hyaluronidase produced			Total			
	Rise	No rise	Total	Percent rise	Rise	No rise	Total	Percent rise	Rise	No rise	Total	Percent rise	Rise	No rise	Total*	Rise	No rise	Total	Percent rise
Treatment A	12	16	28	42.9	3	9	12	25.0	3	3	6		0	2	2	18	30	48	37.5
Treatment B	7	7	14	50.0	0	4	4		3	1	4		0	2	2	10	14	24	41.7
Treatment C	1	0	1		0	1	1		1	0	1		0	0	0	2	1	3	
No treatment	9	11	20	45.0	0	2	2		5	6	11	45.5	0	3	3	14	22	36	38.9
Total	29	34	63	46.0	3	16	19	15.8	12	10	22	54.5	0	7	7	44	67	111	39.6

* Percents not computed for totals with less than 10 cases.

anti-hyaluronidase. There was no significant effect of antibiotic therapy on the formation of anti-hyaluronidase. The same Lancefield type is capable of considerable variation in its capacity to produce hyaluronic acid and hyaluronidase in vitro. The formation of hyaluronidase is shown by a wide variety of types, but the presence of easily detectable amounts of enzyme in 18-hour cultures is uncommon. Table 2 indicates the lack of correlation of time of treatment and anti-hyaluronidase response.

The results of studies on streptokinase are shown in table 5. The supernates of 27% of the strains failed to show the presence of enzyme even when incubated with the fibrin clot for 18 hours. Of 30 infections due to these apparently non-streptokinase-producing organisms, 10 exhibited a significant rise in anti-streptokinase antibody. There was no marked inhibitory effect of antibiotic therapy on the development of anti-streptokinase in patients in this study. Suppression of anti-streptokinase response by penicillin has been reported

TABLE 5.—*Relationship of streptokinase production, group and type, and anti-streptokinase response in treated and untreated patients.*

G & T	Streptokinase produced	Streptokinase not produced	Total
	Number of strains		
A 1	6	2	8
A 3	3	7	10
A 5	17	3	20
A 6	4		5*
A 12	1		1
A 14	15	1	16
A 18	3		3
A 19		1	1
A 30		1	1
A 39	3	1	4
A 42	2		2
A NT	18	7	25
NG	3	2	5
C	4	3	7
G	1		1
?		2	2
Total	80	30	111*

Anti-streptokinase response	Streptokinase produced				Streptokinase not produced				Total			
	Rise	No rise	Total	Percent rise	Rise	No rise	Total	Percent rise	Rise	No rise	Total	Percent rise
Treatment A	16	18	34	47.1	6	8	14	42.9	22	26	48	45.8
Treatment B	8	10	18	44.4	3	3	6		11	13	24	45.8
Treatment C	1		1		1	1	2		2	1	3	
No treatment	13	14	27	48.1	3	5	8		16	19	35	45.7
Total	38	42	80	47.5	13	17	30	43.3	51	59	110*	46.4

Percents not computed for totals with less than 10 cases.
* Streptokinase production unknown for one strain.

TABLE 6.—*The effect of treatment on antibody responses.*

	Rise in 3 antibodies		Rise in 2 antibodies		Rise in 1 antibody		Rise in No antibody		Total	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Treatment A	6	12.5	15	31.2	19	39.6	8	16.7	48	100.0
Treatment B	4	16.7	8	33.3	7	29.2	5	20.8	24	100.0
Treatment C	2						1		3	
No treatment*	12	25.5	15	31.9	11	23.4	9	19.1	47	99.9
Total	24	19.7	38	31.1	37	30.3	23	18.9	122	100.0

Percents not computed for totals with less than 10 cases.
* Includes 11 not seen in sick bay.

by Anderson, Kunkel and McCarthy.[10] Table 2 indicates that delayed treatment was not associated with an increase in antibody formation.

In order to show the effect of treatment on the degree of antibody responses in the patients studied, tables 6 and 7 are presented. The data in table 6 indicate that fewer treated patients exhibited a rise in all three antibodies measured than did those receiving no treatment, but the differences were not significant. The percentages of patients in the treated and nontreated groups exhibiting a rise in two or only one of the antibodies were again not greatly different. The number of untreated patients in table 6 is larger than in the other tables because, included in this group, are some patients on whom serological data were complete but biological characteristics of their infecting strain were not available. Table 7 indicates no significant difference in the degree of antibody rise between the treated and the untreated patients. When the data were further analyzed according to the extent of the treatment as in tables 4, 5 and 6, significant differences again failed to appear.

Statistical analysis applied to these data confirmed the impression that significant differences were not occurring as a result of antibiotic therapy. The low or absent anti-hyaluronidase response to strains producing hyaluronidase or to strains failing to produce it in the absence of hyaluronic acid production was significant ($\chi^2 = 9.71$; $P = 0.002$). Five patients acquired rheumatic fever following a β hemolytic streptococcal infection. The treatment these patients received was as follows: One was given 600,000 units of soluble crystalline penicillin daily for 3 days, the first dose being given on the day of onset of the initial sore throat. One received a single injection of 1,500,000 units of penicillin in oil 2 days after the onset of sore throat; one received 300,000 units of crystalline procaine penicillin G in aqueous suspension daily for 3 days, the first dose being given on the day of onset of the sore

TABLE 7.—*The effect of treatment on the degree of rise for anti-streptolysin O, anti-hyaluronidase and anti-streptokinase.*

Degree of rise*	Anti-streptolysin O			Anti-hyaluronidase			Anti-streptokinase		
	0–1	2–5	6+	0–1	2–5	6+	0–1	2–5	6+
Untreated									
Number	10	13	13	21	11	4	20	12	4
Percent	27.8	36.1	36.1	58.3	30.6	11.1	55.6	33.3	11.1
Treated									
Number	29	31	15	47	24	4	43	29	3
Percent	38.6	41.3	20.0	62.6	32	4	57.3	38.6	4

* These figures indicate the number of tubes in the dilution series beyond the pre-infection or acute phase serum specimen. A one-tube rise in titer was not considered significant.

throat. One received 0.5 g aureomycin 2 days after the onset of the sore throat and 250 mg every 6 hours for 6 doses. One patient received no antibiotics or chemotherapy. The findings of Wannamaker et al[11] strongly suggest that early and adequate antibiotic therapy will prevent the development of rheumatic fever, and it is possible that more vigorous treatment of these individuals would have been effective. The antibody titers in the 5 rheumatic fever patients were high, but no higher than in some of the patients who recovered from streptococcal infection without complications. The organisms responsible for the infection which preceded the rheumatic fever were A-5, A no type, A-14, A-1, A-3 and A-6. A-3 and A-6 were recovered from one patient at a 20-day interval. Three produced hyaluronic acid alone, 3 produced both hyaluronic acid and hyaluronidase; 4 produced streptokinase, and 2 did not. It was assumed that all produced streptolysin.

It seems obvious that there were no consistent similarities in the biologic characteristics of strains causing infections followed by rheumatic fever.

DISCUSSION

In interpreting the findings presented here it should be borne in mind that the bacterial products under consideration are produced by many, if not all, of the Lancefield types of β hemolytic streptococci, and it is therefore improbable that the individuals sustaining these infections were undergoing their first contact with such antigens as streptolysin or hyaluronidase. In explaining the apparent antibody responses during the infections in which the corresponding antigen could not be demonstrated in the infecting strain even by rigorous methods, it is possible that traces of antigenic material undetectable in vitro would be sufficient to produce an anamnestic response in many individuals having previous experience with such antigens. The failure to produce a given toxin in vitro due to nutritional inadequacy of the medium might be advanced as an alternative explanation, as well as lack of specificity of the test itself. In connection with the latter point, it was shown that the interaction of pneumococcal polysaccharide or crystalline ovalbumin with its specific antibody failed to inactivate hyaluronidase when it was present during the formation of the precipitate, indicating that the inhibition of hyaluronidase is probably specific.

The suppressive effect of treatment on the antibody response in this group of patients seemed to be minimal and by no means consistent. It is possible that the examination of larger numbers of patients would result in significant differences. The frequently observed antibody response following prompt antibiotic therapy is not surprising, and may again be a manifestation of a large response to a minimal antigenic stimulus as a result of a recall mechanism. In addition, if one considers the incubation period of streptococcal infections of 24 to 48 hours, plus a delay of another 24, 48 or even 72 hours before administration of treatment with an antibiotic, there would be a minimal interval of 2 or 3 days during which antigenic stimulation could occur. Very few of these patients were treated within 24 hours of the onset of symptoms. In connection with antibody response to the type specific M substance of Lancefield, one would not expect such recall

11. Wannamaker, L. W., Rammelkamp, C. H., Denny, F. W., Brink, W. R., Houser, H. B. Hahn, E. O. and Dingle, J. H. 1951, Prophylaxis of acute rheumatic fever by treatment of the preceding streptococcal infection with various amounts of depot penicillin. Am. J. Med. **10**: 673–695.

mechanism to appear. The effect of treatment on this antibody response has been studied and is said to be marked.[12] Attempts to confirm this finding are in progress.

No correlation of Lancefield type or biological characteristics of the infecting organism and clinical response could be established. Patients infected by apparently identical strains exhibited differences in clinical response, and those who had illnesses of similar severity and duration were often infected by organisms differing in the characteristics studied. Hyaluronic acid and hyaluronidase do not appear to be produced simultaneously by the same organism; there is evidence[13] that hyaluronidase-producing variants arise during prolonged incubation, particularly under anaerobic conditions. Whether this variation occurs in vivo is not known. We are unable to explain the apparent paradox of low anti-hyaluronidase response in patients infected with organisms producing that enzyme in the absence of hyaluronic acid. In most cases these strains produce the largest amounts of hyaluronidase. It might be concluded that such strains are not necessarily infective and may be isolated by chance from nonstreptococcal lesions. However, this is not supported by the fact that of 19 patients showing this variety of organism, 17 exhibited a significant rise in antibodies to streptolysin O, streptokinase, or both.

12. Daikos, G. and Weinstein, L. 1951, Streptococcal bacteriostatic antibody in patients treated with penicillin. Proc. Soc. Exper. Biol. & Med. **78**: 160–163.

13. Sallman, B. 1951, The process of hyaluronidase formation by hemolytic streptococci. J. Bact. **62**: 741–746.

The same more or less unanswerable question of etiology can be raised in connection with any throat infection from which β hemolytic streptococci have been isolated. One might particularly suspect those in which the organism was not classified in group A, or, although belonging to Lancefield's group A, could not be typed. Thirty-five of forty such instances were again associated with significant increase in antibodies to one or another of the organism products tested.

SUMMARY

The relationship between the ability of a given strain of β hemolytic streptococcus to produce streptokinase, hyaluronidase and hyaluronic acid and the antibody response to streptolysin O, streptokinase and hyaluronidase in individuals infected by these strains has been studied. Failure to show a rise in antibody to a given streptococcal product was observed although the infecting organism elaborated that product. Moreover, antibody increases were frequently observed in patients whose infecting organism could not be shown to give rise to the corresponding antigen. The implications of this are discussed.

There was no consistent relationship between Lancefield type and the formation of streptokinase, hyaluronic acid and hyaluronidase. Seventeen percent of the strains produced hyaluronidase alone, 76% produced hyaluronic acid, and 73% produced streptokinase.

Antibiotic therapy appeared to have only a slight influence in inhibiting antibody response to streptolysin O, streptokinase, and hyaluronidase in the patients studied.

THE ROLE OF THE LAUNDRY IN THE RECONTAMINATION OF WASHED BEDDING

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During the course of a study of the use of ethylene oxide gas (Carboxide*) as a routine procedure for sterilizing hospital bedding and equipment, baseline observations were made on the bacterial content of freshly laundered sheets, cotton and woolen blankets and other textiles.[1] The majority of strains isolated were β and nonhemolytic staphylococci and α streptococci. As these organisms are generally considered to be of human or animal origin, it seemed important to investigate the laundry process and environment for a possible explanation of their presence in the washed and ironed fabrics. During the investigation extensive sampling of the air, water and textiles was done for the presence of bacteria during the fall and winter months of 1950, 1951 and 1952.

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* Carboxide is a commercial preparation of 10% ethylene oxide and 90% carbon dioxide.

1. Church, B. D. and Loosli, C. G. A study of the practical application of ethylene oxide gas (Carboxide) as a sterilizing procedure for hospital supplies and equipment. To be published.

MATERIALS AND METHODS

Laundries.—The study was carried out in two laundries. Laundry A was a small but complete unit operated by the maternity section of the hospital. Laundry B was a commercial laundry which served the rest of the hospital on a contract basis. The physical set-up, machinery and laundry procedure (soap, time, etc.) in the two laundries were essentially the same. All the operations were carried on in one large room with no partitions. In laundry A, shown in figure 1, the used linen was taken from the collection room and sorted near the washers. The textiles received at laundry B were already sorted and introduced into the wash wheels directly from laundry bags. The specifications for washing met those recommended by the American Laundry Institute[2] and the American Hospital Association,[3] with the exception that in laundry B the water for washing cotton fabrics was heated only to 160 F.

There was no effort to control the ventilation or air currents in either laundry. During the cold winter months the windows were closed and air entered through the doors leading to and from the laundry. In laundry A there was a marked down-draft from the laundry chute out into the large room. During the summer months when the windows were open and the fans were on, the number of bacteria isolated from the air varied greatly and had no particular relationship to laundry activity. There was obviously and always much more dust on the surfaces, including the floor, equipment, etc., in laundry B than in laundry A.

2. American Institute of Laundering. The sanitary aspects of commercial laundering. Bull. no. 53, July, 1942. Joliet, Illinois.
3. Barnes, M. E. October 1948, The public health aspects of the hospital laundry, Manual on Hospital Laundry Operation, Am. Hosp. Assoc., pp. 121–137.

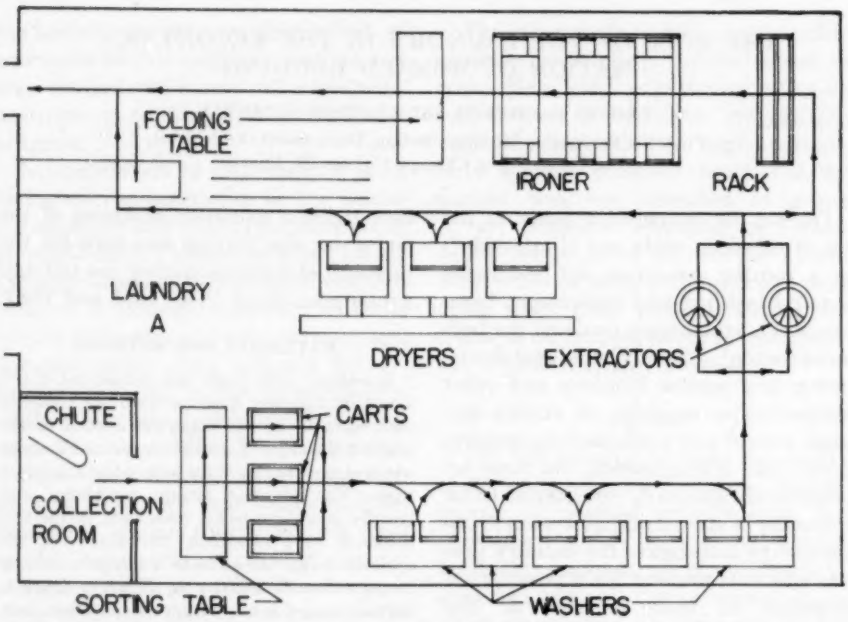

FIG. 1.—Diagrammatic sketch of the layout in laundry A. Note the sorting table in the open room and the position of the extractors.

Sampling air.—Folin-bubbler samplers[4,5] were employed for the collection of bacteria from the air. They were placed at different sites in the laundry, and air was drawn through them at the rate of one cubic foot per minute. Usually a 5-minute sample was taken. Aliquots of the broth were cultured, using beef heart infusion agar and 5% sheep blood. Bacteria were estimated on the basis of number per cubic foot of air.

Sampling bedding.—The *Folin-bubbler sampler* with a funnel attached was also employed for collecting bacteria from textiles.[5] The material to be sampled was placed in a three-square-foot quilting hoop. Smaller items were placed in a one-square-foot hoop. With air flowing through the bubbler at the rate of one cubic foot per minute the funnel was gently brushed over the surface of the cloth within the hoop areas. Aliquots of the broth were then cultured as described above. The bacterial counts were estimated as the number removed from one square foot of surface in one minute. The bedding was sampled in a closed 640 cubic foot experimental room which had been cleaned by swabbing the walls with 70% alcohol and introducing filtered air. On sampling the air before the bedclothes were analyzed it was generally found to be free of organisms.

The Waring blendor, in the latter part of the study, was employed to obtain more quantitative estimates of the number of bacteria in the "clean" and used bedding. One square foot of the textile to be examined was cut with sterile scissors into small pieces and placed in a Waring blendor into which 300 ml of sterile beef heart infusion broth had been added. It was then run at 12,000 r.p.m. for 3 minutes. Aliquots of the broth were cultured as described above. Bacterial counts were estimated as the number per square foot of sampled cloth.

After each bedding item was sampled, the blendor was washed in soap and water, rinsed in 70% alcohol, inverted, and allowed to dry in air for approximately 2 hours. Sterile broth was spun in the blendor and cultured before the next textile was triturated. In most instances the con-

4. Lemon, H. M. 1943, A method for collection of bacteria from air and textiles. Proc. Soc. Exper. Biol. & Med. **54**: 298–301.
5. Loosli, C. G., Lemon, H. M., Wise, H. and Robertson, O. H. 1948, Studies on the transmission and control of respiratory disease within army barracks. I. Hemolytic streptococcal contamination of the environment. J. Infect. Dis. **82**: 59–71.

trol, broth samples from the blendor, showed no growth. Occasionally 0.1 to 0.5 organisms per ml were recovered.

Sampling water.—Samples of wash and rinse water, taken at various stages during the laundry procedure, and from the extractor pipes were cultured as described above. The bacterial counts were estimated as the number of organisms per ml.

Bacteriological procedures.—Standard bacteriological diluting, duplicate plating and counting procedures were carried out. All cultures from Folin-bubbler and Waring blendor samples were incubated at 37 C for a period of 72 hours. Periodic inspection of all cultures was made at 24- and 48-hour intervals. Identification of bacterial species was conducted according to methods recommended in Bergey's Manual of Determinative Bacteriology (6th edition).

Alpha hemolytic streptococci and beta hemolytic and nonhemolytic staphylococci were studied in detail because of their frequent occurrence, their possible etiological significance, and the necessity for distinguishing the alpha streptococci from pneumococci. Colonies of streptococci were examined for their reaction with inulin, ox bile, starch, gelatin, salicin, mannite, lactose and growth at 50 C. Staphylococci were subcultured on tryptose agar for pigmentation and inoculated into sterile 10% citrated human plasma for coagulase determinations.

RESULTS

Bacterial counts from freshly laundered, unused bedding.—The initial studies were begun employing the Folin-bubbler sampler for recovering bacteria from textiles. The number of organisms recovered per square foot of surface from different pieces of bedding is shown in table 1. Clean pillowcases and bedspreads which had been on unused beds for several days showed a greater number of organisms than did those removed directly from the paper wrapper. This increase was considered to be due to bacteria-laden dust particles settling on the exposed spreads and pillowcases. Sheets and cotton and woolen blankets protected by the bedspread showed no such increase.

Variation in bacterial counts from soiled bedding.—As has been shown by Lemon[4] and Hamburger and associates,[6] it was found that the bacterial content of a given piece of used bedding varied greatly, depending on where and in what position it was placed on the bed in relation to the patient. As shown in table 2, the head end of the bedspreads, bottom sheet and blankets yielded more organisms than did the foot end. The difference in numbers found at the head compared to the foot in case of blankets was not as great as in the case of the bottom sheets and spread. Pillowcases, gowns, and pajama jackets also yielded large numbers due to the direct exposure to the nose and mouth secretions and soiled hands of patients.

Comparison of methods for determining the number of bacteria in soiled and

TABLE 1.—*Numbers of organisms isolated with the Folin-bubbler sampler from unused bed linen immediately removed from the laundry and after being on unused beds from 2 to 10 days.*

Textiles	From laundry		From unused beds	
	Number of tests	Mean number of organisms/ft²	Number of tests	Mean number of organisms/ft²
Pillowcase	20	160	25	520
Bedspread	22	80	12	540
Sheets	20	120	9 (top)	130
			9 (bottom)	120
Wool blankets	21	1290	3	1380
Cotton blankets	19	1500	3	1800

TABLE 2.—*Variation in the number of organisms isolated with the Folin-bubbler sampler from different areas of used bedding.*

Textiles	Number of tests	Mean number of organisms/ft²			
		Topside		Underside	
		Head	Foot	Head	Foot
Bedspread	16	18,000	5000	100	200
Pillowcase	14	68,000		300	
Top sheet	15	40	70	8000	900
Bottom sheet	15	38,000	1000	500	300
Mattress cover	8	70	80	20	10
Gown	10	25,000		200	
Pajama jacket	8	38,000		80	
Wool blanket	15	6,200	1800	1400	1300
Cotton blanket	15	4,300	2000	1600	1800

6. Hamburger, M., Jr., Green, M. J. and Hamburger, V. G. 1945, The problem of the "dangerous carrier" of hemolytic streptococci. I. Number of hemolytic streptococci expelled by carriers with positive and negative nose cultures. J. Infect. Dis. **77**: 68–81.

TABLE 3.—*The numbers of bacteria isolated from used and unused (freshly laundered) bedding employing the Folin-bubbler sampler, compared with the number isolated with the Waring blendor.*

Textiles	Folin-bubbler	Total number of bacteria/ft²		Mean ratio
		Waring blendor	Blendor / Bubbler	
Unused				
Pillowcase	30	2,700	90	
Topsheet	30	2,300	77	
Topsheet	20	1,300	65	
Cotton blanket	266	17,500	66	76
Cotton blanket	317	26,500	84	
Wool blanket	3,100	224,000	72	
Wool blanket	4,200	326,000	78	
Used				
Topsheet	1,200	166,000	14	
Topsheet	3,700	124,000	34	
Topsheet	8,300	130,000	16	
Cotton blanket	4,500	113,000	25	29
Cotton blanket	5,400	188,000	35	
Wool blanket	9,400	383,500	41	
Wool blanket	31,300	1,860,000	40	
Used				
Pillowcase	63,000	156,000	3	
Pillowcase	24,000	64,500	3	
Pillowcase	62,000	210,000	4	4
Bottom sheet	32,400	90,200	3	
Bottom sheet	53,200	303,800	6	
Bottom sheet	41,000	150,000	4	

TABLE 4.—*Approximate percent of species of bacteria isolated from hospital bedding (Waring blendor sampling).*

	Used bedding	Unused bedding
Number of ft² samples	124	126
Mean total organisms/ft²	473,290	5854
Microorganisms	Percent	Percent
Staph. albus	2	30
Staph. aureus	1	10
Streptococcus salivarius	3	30
Streptococcus thermophilus	1	20
Neisseria catarrhalis	3	0
Escherichia coli	10	0
Sarcina lutea	5	1
Proteus vulgaris	2	0
Bacillus mycoides	35	2
Bacillus subtilis	10	3
Others	12	4

"clean" bedding.—Because it was recognized that the Folin-bubbler sampler with the funnel attachment removed only the loosely attached organisms from the surfaces of bedding, a more quantitative method (the Waring blendor) for determining the total number of bacteria from a given piece of bedding was utilized. Table 3 shows that the Waring blendor yielded bacterial counts in some experiments several hundred times greater than did the Folin-bubbler sampler. The ability of the blendor to remove greater numbers of bacteria was seen especially in the case of clean, unused bedding and to the least extent on highly soiled sheets and pillowcases. This would be expected because more easily detached bacterial particles exist on the used bedding than on the clean freshly laundered materials.

Kinds of organisms found on hospital bedding.—A wide variety of organisms was isolated from freshly laundered and used bedding. Some of the more frequently isolated strains are shown in table 4. Bacterial counts on soiled bedding, although high, yielded relatively small numbers of staphylococci and streptococci. Hemolytic streptococci were isolated only from the bedding of known cases of streptococcal disease and are not included in table 4. The bacterial count on ironed bedding was greatly reduced but the majority of organisms isolated were *Staphylococcus albus* and *aureus* and α and nonhemolytic streptococci. Many of the strains of *Staph. aureus* showing β hemolysis were coagulase positive.

Laundry air.—Numerous air samples for bacterial content were obtained at different sites in laundries A and B during the course of the day's activities. As seen in figure 2, the number of bacteria per cubic foot of air at different sites varied greatly but in general were parallel. At most sites, as seen in figure 2, there was a higher bacterial content of the air of laundry B than A. The largest numbers of air-borne bacteria in both laundries were found near the sorting table, by the water extractors, while they were in operation, and near the rotary dryers. The lowest counts were near the washing machines when they were being unloaded and near the ironer. Considerable numbers were isolated from the air near the folding table. The actual number of bacteria per cubic foot of air sampled at the same sites in laundries A and B during routine laundry activities is shown in tables 5 and 6.

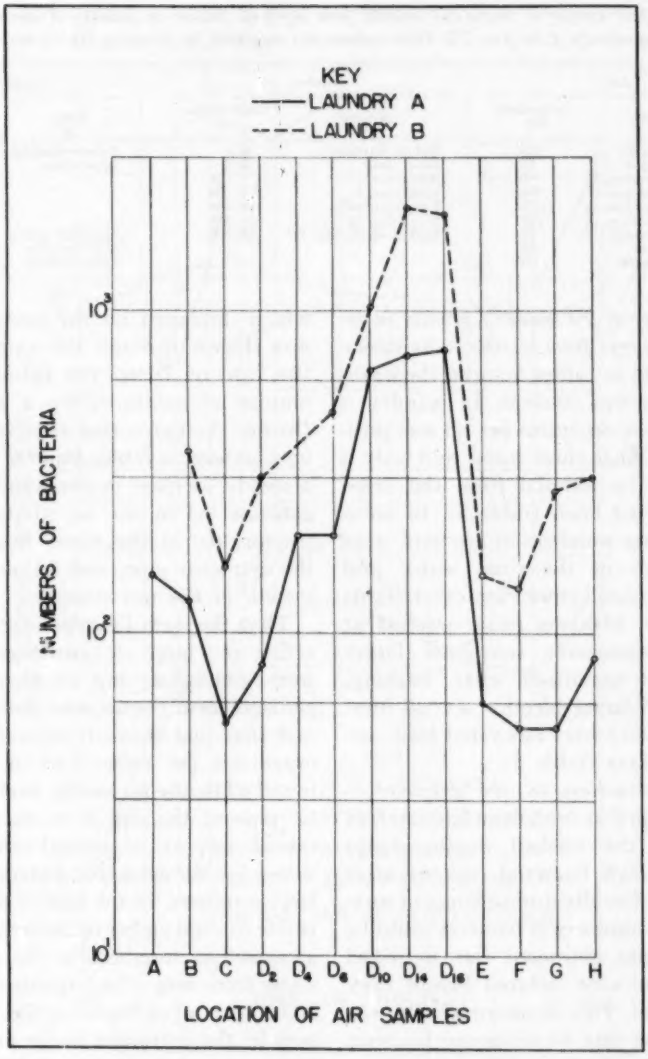

FIG. 2.—Total number of bacteria per cubic foot of air sampled at specific sites in laundries A and B during routine activities.

A = Sorting table
B = Loading washing machine
C = Unloading washing machines
D_2 = Near extractor, 2 minutes
D_4 = Near extractor, 4 minutes
D_6 = Near extractor, 6 minutes
D_{10} = Near extractor, 10 minutes
D_{14} = Near extractor, 14 minutes
D_{16} = Near extractor, 16 minutes
E = Near extractor, off
F = Near ironer
G = Near dryer
H = Near folding table

TABLE 5.—*The number of organisms isolated from different sources in laundry A during maximum sorting activity, 2:30 p.m. The Folin-bubbler was employed for sampling the air and linen.*

Air		Water		Linen	
	Location	Organisms /ft³	Source	Organisms /ml	Step	Organisms /ft²
(A)	Sorting table	130	Before washing	0.1	Before washing	2000
(B)	Near washer	58	Final rinse	0.3	After rinse	10
(D_2)	Near extractor, 2″	80	Extractor, 2″	10		
(D_6)	Near extractor, 6″	200	Extractor, 6″	2,600		
(D_{10})	Near extractor, 10″	670	Extractor, 10″	15,000		
(D_{14})	Near extractor, 14″	720	Extractor, 14″	26,000		
(E)	Near extractor (off)	58	End of extraction, 16″	28,000	After extraction	2300
(F)	Near ironer	50			After ironing	30
(H)	Folding table	86			After folding	150

Efficiency of the laundry process in removing bacteria from textiles.—As shown by the data in tables 5 and 6 the washing process was efficient. In laundry A less than one organism per ml was present in the final rinse water and only a few could be isolated from the dried non-extracted linen (table 5). In laundry B larger numbers of bacteria were found both in the rinse water and washed clothes before extraction (table 6). Woolen blankets being washed at lower temperatures contained larger number of organisms after washing. With the Waring blendor several hundred thousand were recovered from air-dried blankets (table 3).

Recontamination in the extractor.—As was found in both laundries (tables 5 and 6) the washed textiles again showed a high bacterial content after extraction. Usually during hours of sorting greater numbers of bacteria could be isolated from extracted but unironed sheets than were isolated before they were washed This recontamination was found to be due to air-borne bacteria which impinged on the textiles as air was drawn through the extractors at the rate of 70 to 100 cubic feet per minute as measured by a velometer During the extraction time of 16 minutes, as seen in tables 5 and 6, there was a steady increase in the number of organisms (a) in the air above the extractor, (b) in the water issuing from the extractor pipe, and (c) as has been stated, in the wet textiles.

That the open lid type extractor was acting as a large air centrifuge with the wet bedding serving to filter the organisms form the air was shown by the fact that just above it several hundred organisms per cubic foot of air were found while the air issuing from the outlet pipe at the end of extraction contained only an occasional one. At the same time the extracted water contained large numbers. In the case of laundry A (table 5) the number of bacteria steadily increased in numbers in the extracted water from only a few organisms to over 30,000 per ml of liquid, while the numbers in the extractor water in laundry

TABLE 6.—*The number of organisms isolated from different sources in laundry B during maximum sorting activity, 2:30 p.m. The Folin-bubbler was employed for sampling the air and linen.*

Air		Water		Linen	
	Location	Organisms /ft³	Source	Organisms /ml	Step	Organisms /ft²
(A)	Sorting table	350	Before washing	200	Before washing	38,000
(B)	Near washer	135	Final rinse	250	After rinse	350
(D_2)	Near extractor, 2″	300	Extractor, 2″	12,200		
(D_6)	Near extractor, 6″	500	Extractor, 6″	45,400		
(D_{10})	Near extractor, 10″	1040	Extractor, 10″	601,000		
(D_{14})	Near extractor, 14″	2150	Extractor, 14″	1,080,000		
(E)	Near extractor (off)	150	End of extraction, 16″	1,940,000	After extraction	165,000
(F)	Near ironer	140			After ironing	250
(H)	Folding table	300			After folding	1,140

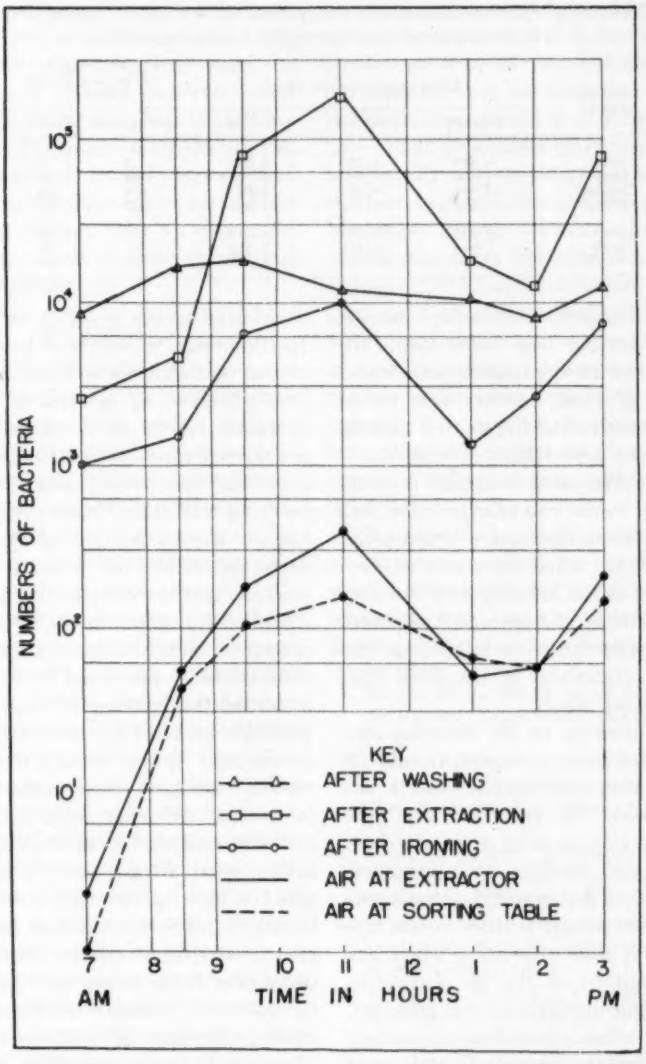

FIG. 3.—Numbers of bacteria isolated from bedding and air in laundry A during different periods of a day's activity. Maximum sorting occurred from 10:00 to 11:00 a.m. and at 3:00 p.m.

B (table 6) increased from a few hundred to over a million per ml of fluid. During this process in laundry B the number of bacteria increased in the textiles from a few to over a hundred thousand organisms per square foot of cloth. By using the Waring blendor in Laundry A, greater numbers of organisms were isolated from the washed bedclothes both before and after water extraction, as shown in table 7. The data in this table also show that the number of organisms isolated from (a) the air, (b) textiles before and after washing and ironing, and (c) extractor water was in direct relation to the ex-

TABLE 7.—*Numbers of organisms isolated from different sources in laundry A during a day's operation. The Waring blendor was employed for sampling the linen.*

Time in hours	Sorting activity	Linen (organisms/ft²) after:			Air (organisms/ft³) above:		Water (organisms /ml) extractor
		Washing	Extracting	Ironing	Sorting table	Extractor	
A.M.							
7:00	None	9,587	2,550	1,012	0	2	160
8:30	Begin	14,400	4,500	1,357	38	48	660
9:30	Sorting	15,600	79,662	6,097	104	184	14,060
11:00	End of	11,812	292,122	11,662	180	376	36,800
P.M.							
1:00	None	11,137	18,150	1,252	58	50	490
2:00	Begin	7,012	13,422	2,512	56	56	284
3:00	End of	12,750	82,912	7,395	128	164	24,200

tent of sorting activities in the laundry. When no sorting was being done the least number of organisms was found while the greatest number was found just before the end of the sorting periods at 11:00 a.m. and 3:00 p.m. (fig. 3).

That the increased bacterial content in the linen at the end of extraction was due to air-borne organisms is indicated by the fact that when there was no sorting activity in the laundry and the bacterial content of the air was low, there was at the same time no increase in the number of organisms in the linen (fig. 3) or extractor water.

Effect of ironing on the bacterial content of washed linen.—As seen in table 7, a considerable number of viable organisms could be isolated from the sheets after ironing with a standard six roll chest-type machine at a temperature of 350 F. The bacterial content was highest in the ironed textiles in the late morning and mid afternoon when the bacterial content of the air, extractor water and unironed fabrics was greatest, and lowest when there was no sorting activity. Thus, the increased numbers of organisms in the ironed bedding may be attributed to the inability of the ironer to eliminate the bacterial load built up in the textiles during water extraction.

Mechanisms of survival of organisms in cotton fabrics after ironing.—It was considered that many of the organisms surviving the ironing procedure might be those which had been dispersed into the air during sorting of the unlaundered materials and filtered on to the washed textiles during the process of water extraction. Such bacteria dispersed in dried mucous particles possibly could be protected from the lethal action of the heat by this covering. To test this possibility cultures of *Staph. aureus* and *Streptococcus salivarius* previously isolated from used bedding in several different experiments were grown, resuspended in distilled water, saline, nutrient broth or 1% mucin and sprayed in a bacteria-free room and allowed to settle for one-half hour on to steam sterilized sheets. Square foot samples of these sheets were then tested for bacterial content before and after ironing. The results are shown in table 8. The ironed sheets on which had been sprayed organisms suspended in dis-

TABLE 8.—*The effect of the "bacterial coating" on the survival of organisms on artificially contaminated sheets after ironing (Waring blendor samples).*

Microorganisms suspended in	Bedding contaminated with							
	Staph. aureus				*Strep. salivarius*			
	Number of tests	Before ironing	Number of tests	After ironing	Number of tests	Before ironing	Number of tests	After ironing
Distilled water	6	291,000	8	585	2	146,850	8	453
Broth	4	347,000	10	566	4	155,000	10	612
1% mucin	4	351,000	10	2412	4	154,000	10	1927

tilled water, saline or broth yielded approximately the same number after ironing. However, approximately three to four times more bacteria were recovered from sheets sprayed with organisms suspended in 1% mucin after the ironing process. These data would indicate that in addition to the inherent nature of the bacterial cell to resist heat, an important factor is the extraneous dried mucous coating surrounding it.

DISCUSSION

In 1938 Arnold[7] made a study of the efficiency of the laundry procedure in removing bacteria from soiled clothes. His findings have served as the basis for recommended procedures of the American Institute of Laundering[2] and the American Hospital Association.[3] The data in the present study show that the laundry procedures were generally efficient in removing bacteria from used textiles as Arnold had shown. They do not, however, support his observations that the extraction and ironing processes remove or kill all the residual bacteria. On the contrary, they show that during the periods of laundry sorting and operation the extraction process, when open-lid centrifuge extractors are employed, is responsible for recontamination of the washed materials to a high degree. The ironing process, although efficient, was unable to kill many of these organisms which by-passed the washing process via the air only to find their way back into the textiles through the water extractor or from direct settling out on blankets hung up to dry. One possible factor contributing to the survival of these organisms during the ironing process was the dried mucus covering the bacterial cell.

Although concern for the health of the workers in laundries has been emphasized by Barnes[3,8] and Heeren and associates,[9] no mention is made in their studies or that of Arnold[7] concerning the possible role of air-borne bacteria in the recontaminating of hospital textiles. That this mode of contamination is generally not considered is shown by the fact that it is not mentioned in the Manual on Hospital Laundry Operation of the American Hospital Association, October, 1948,[3] and the American Institute of Laundering Bulletin no. 53, 1942[2] on the sanitary aspects of commercial laundering. Likewise, it is not mentioned in more recent reports concerning the aspects of environmental sanitation in hospital construction programs[10] and operating hospital laundries.[11,12]

That hospital bedding may serve as a principal secondary reservoir for agents pathogenic for man in military barracks and hospital wards is well known.[5,6,13–17] In some situations these

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reservoirs may serve as one of the important sources of organisms causing secondary streptococcal and staphylococcal infections of wounds, burns, eczema, streptococcal tonsillitis and skin infections in children's wards, nurseries and maternity hospitals.[14–19] In the case of staphylococcus infections in hospitals many of the strains are shown now to be resistant to penicillin and other antibiotics.[20,21]

It is not inconceivable that so-called "clean linen" which has become recontaminated with air-borne organisms dispersed from unwashed bedding may be the source of initiating serious infections when placed again on hospital beds.[17,22] A good laundry process for removing bacteria and other pathogenic agents becomes of little importance if the environment in which it is carried out serves only to recontaminate the bedding with the same organisms.

The recontamination by air-borne bacteria of washed bedding as it passes through the water extractors, as it hangs in the laundry to dry and while it is being folded for transfer back to the hospital ward or home, is a potential public health hazard. This should be recognized by the appropriate authorities and measures instituted to prevent it by proper laundry management, construction and ventilation control.

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SUMMARY

An extensive bacteriological study in two laundries (one hospital and one commercial) of the air, wash and rinse water, and textiles before washing, and after rinsing and ironing, was made to determine the source of organisms found in the laundered materials. It was found that the laundry processes were efficient in removing bacteria from the fabrics during washing, but the materials became recontaminated during water extraction in the spin dryer or while hanging in the laundry to dry.

This was shown by the fact that the air during the sorting of linen and other activities became highly contaminated with bacteria, which settled out on woolen blankets hanging in the laundry and on fabrics at the folding table. In addition, large numbers of air-borne bacteria were drawn through the spin dryers during the process of water extraction and impinged on the washed textiles to a high degree. Although the ironing process killed large numbers of these organisms, many survived.

As the surviving organisms were mainly nonhemolytic and α streptococci and *Staphylococcus albus* and *aureus*, which are potential human pathogens, the findings in this study become of public health importance. The public health implications were discussed and measures were suggested for preventing recontamination of washed bedding in the laundry through reevaluation of laundry procedures, construction and ventilation control.

THE RELATION OF THE TRICARBOXYLIC ACID CYCLE TO BACTERIAL INFECTION

I. THE EFFECT OF MALONATE ON *SALMONELLA TYPHIMURIUM* INFECTIONS IN MICE

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In recent experiments, Ackermann (1951a) has attempted to relate host metabolism with response to virus infection. He found that injections of sublethal amounts of sodium fluoroacetate in mice result in an accumulation of citric acid in the lungs, due to a blockage of the tricarboxylic acid cycle, and, under the same conditions, inhibit the synthesis of influenza A virus administered by nasal insufflation. He previously had reported (1951b) that viral synthesis by tissues growing in vitro is inhibited by sodium malonate and by antimycin A, an antibiotic that inhibits the enzyme succinic dehydrogenase (Ahmad et al, 1950). A similar inhibition in the synthesis of the Lansing strain of poliomyelitis virus in mice given sublethal doses of fluoroacetate was reported by Ainslie (1952). If, according to Cohen (1949), viruses reproduce at the expense of the synthetic abilities of the parasitized cells, then the results of Ackermann and of Ainslie tie the Krebs cycle to this process and imply that energy made available by reactions of the cycle is utilized for synthesis of virus protein. These workers did not, however, determine whether the reduction of viral propagation following sublethal doses of fluoroacetate, or of any other inhibitor, decreased the mortality of mice infected with influenza virus. This was done quite recently by Mogabgab and Horsfall (1952), who found insignificant differences in survival or in virus synthesis over a period of a day or longer in mice or chick embryos poisoned with fluoroacetate and infected with the viruses of the PR 8 strain of influenza A, the Lee strain of influenza B, mumps virus, or pneumonia virus of mice (PVM).

Possibly related to the above observations are those of Kalter and Tepperman (1952), who report a reduction in the synthesis of influenza virus in mice subjected to the hypoxia of a simulated altitude of 30,000 feet. The greatest difference in virus production between control and hypoxic mice was obtained 15 hours after the animals were infected. This suggests that hypoxia lowers viral propagation because of some change in metabolism which, in turn, reduces the synthetic capacity of the cells. The same interpretation explains why some strains of mice adapted to a simulated altitude for three weeks are more resistant than control mice to infection with certain concentrations of influenza A virus, even though the altitude-adapted mice are maintained at normal atmospheric pressures for the entire postinfection period (Berry and Mitchell, unpublished). The greater resistance of the altitude-adapted mice to influenza is small but statistically significant, whereas similar mice are more susceptible than control mice to infection with

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Salmonella typhimurium (Berry and Mitchell, 1951).

From these experimental facts the following relationships are brought into focus: (a) inhibition of the citric acid cycle by a metabolic poison reduces the synthesis of influenza virus in the lungs and of poliomyelitis virus in the central nervous system of mice; (b) hypoxia of a simulated high altitude reduces the synthesis of influenza virus in the lungs of mice; (c) adaptation to a simulated high altitude for three weeks makes mice, after they are returned to normal pressures, statistically *more resistant* than control mice to infection with influenza virus; and (d) mice similarly adapted to altitude and then kept at normal pressures are *more susceptible* to infection with *S. typhimurium* than control mice. The question arises as to whether mice are more susceptible to infection with *S. typhimurium* when the citric acid cycle is inhibited than are normal mice. Experiments were designed to answer this question and the present report describes the results obtained with sodium malonate as inhibitor. Elsewhere (Berry and Mitchell, 1953b), results with fluoroacetate, arsenite, citrate, and succinate are presented. A preliminary paper has appeared (Berry and Mitchell, 1953a).

Malonic acid, or its sodium salt, has been used as a metabolic inhibitor ever since Quastel and Whetham in 1925 found that the reduction of methylene blue in a Thunberg tube by a suspension of *Escherichia coli* in the presence of succinic acid was blocked by malonic acid. Its role as a competitive inhibitor of succinic dehydrogenase has been elucidated in the intervening years. Krebs et al (1952), for example, recently found that the oxidation of succinate by baker's yeast is about 50% inhibited when the concentration ratio of succinate to malonate is 60. The action of malonate in vivo seems to be more complicated. This can be seen when injections are made in rats or rabbits because, as Krebs, Salvin and Johnson (1938) showed, there follows a urinary excretion of succinic acid and, to a lesser extent, of α-ketoglutaric and citric acids. The excretion of these acids is attributed to the inhibition of enzymes concerned with their breakdown. A possible way in which malonate may inhibit these enzymes has been pointed out by Pardee and Potter (1949) in their effort to explain how it decreases the oxidation of oxaloacetate. Malonate apparently forms a complex with free and bound magnesium, thereby lowering the concentration of the ion, and inhibiting enzymes requiring its presence as a co-factor or activator. Therefore, injections of malonate into an animal lead to the inhibition of an unknown number of enzymes, one consequence of which is the demonstrable increase in concentration of succinic acid and citric acid in the blood and organs (Busch and Potter, 1952a). Another result, described in this paper, is the impressive reduction in survival time of mice artificially infected with the organisms of mouse typhoid followed by injections, at hourly intervals, of sublethal amounts of malonate, as compared to the survival time of mice similarly infected and injected at the same time intervals and with the same volume of normal saline.

MATERIALS AND METHODS

Sodium malonate.—Disodium malonate, Eastman, was dissolved in a sterile solution of normal saline (0.9% sodium chloride) in such concentration that the desired number of milligrams was contained in 0.5 ml of the final solution. Dosages of 10, 15, 20, and 40 mg per mouse per injection were employed. A fresh solution of malonate was prepared each day it was used. All injections were given intra-abdominally.

Bacterial suspension.—Mice were infected with a strain of *S. typhimurium* isolated in 1949 by one

of us (RBM) from a mouse which had died of typhoid. This strain has been used repeatedly for nearly 3 years with no apparent change in virulence or cultural characteristics. Reproducible mortality data for groups of mice infected from time to time throughout this period have been obtained with suspensions prepared in the following manner: Brain-heart infusion broth (Difco) is inoculated and then incubated for 18 hours at 37 C. One-tenth ml of this culture is transferred to 9.9 ml of the same medium and incubated at the same temperature for 5 hours. Growth studies at intervals have shown that a 5-hour culture contains about 750 million bacteria per ml. This culture is diluted with sterile normal saline until the desired number of cells is contained in 0.5 ml. The number varied in these experiments from 250,000 to 5000 bacteria. The suspension was always prepared immediately before use and all injections were given intraperitoneally.

General.—Young adult female mice of the CF-1 strain were used in these experiments. They weighed from 20 to 25 g. The animals were kept in small galvanized iron cages containing wood shavings, with about ten mice per cage. Cages were sterilized in the autoclave after they had housed infected mice. The animals were fed Purina dog chow ad libitum and water was available at all times.

RESULTS

Effect of malonate on infection with S. typhimurium

Table 1, column 3, gives the mortality data of 10 mice infected with 250,000 cells of *S. typhimurium*, followed immediately by an injection containing 20 mg sodium malonate. The malonate injections were repeated at hourly intervals until a total of 8 injections (160 mg) was administered. It is seen that 5 of the mice died within 7 hours and the remaining 5 were dead the following morning. These results should be compared with those for the control mice, which received the same number of bacteria and the same number of injections of normal saline (column 6). The first of these mice died the 3rd day after infection and the last died on the 6th day. Mice receiving the malonate but no bacteria (column 4) survived, except for the death of one mouse on the 6th day, and this death was probably the result of accidental infection. It is therefore apparent that bacteria and malonate combine to produce in mice a remarkably fast acting "infection."

The apparent need for repeated injections of malonate in order to maintain a block of the tricarboxylic acid cycle in the infected mice was based on reports in the literature. Lifson and Stolen (1950) injected C^{13}-carboxy-labeled malonate into mice and then recovered from 20 to 32% of the labeled carbon in the respiratory carbon dioxide within 6 hours. Lee and Lifson (1951) injected isotopic malonate into rats and isolated labeled succinic acid from the urine. Busch and Potter (1952b) used the chromatographic technique of Busch, Hurlbert and Potter (1952) to find 55% of the injected malonate eliminated in the urine of rats within about 6 hours. From this combined evidence, it is apparent that the rat and the mouse are capable of eliminating and metabolizing malonate. As direct proof, Busch and Potter (1952b) show that a single injection of malonate

TABLE 1.—*Survival of mice infected with* S. typhimurium *and injected hourly with malonate or with saline (10 mice per group).*

Time interval in hours	Total number injections	Experimental 20 mg (½ ml) malonate hourly			Control ½ ml saline 250,000 bacteria
		250,000 bacteria	No bacteria	250,000 heat-killed bacteria	
(1)	(2)	(3)	(4)	(5)	(6)
0–1	1	10	10	10	10
1–2	2	10	10	10	10
2–3	3	10	10	10	10
3–4	4	10	10	10	10
4–5	5	9	10	10	10
5–6	6	8	10	10	10
6–7	7	5	10	10	10
7–24	8	1	10	10	10
24–36	8	0	10	10	10
36–48	8		10	10	10
48–60	8		10	10	10
60–72	8		10	10	2
72–96	8		10	10	1
96–120	8		9	10	1
120–144	8		9	9	0

causes a peak succinate concentration in the blood within about one hour, and within another hour or two the succinate concentration is back to normal. Repeated injections in rats of malonate at a dose level about double that used in our mice, on a per kg of animal basis, caused an additional increase in the succinate content, as well as in malonate content, of blood and tissues. The organs in which succinic acid and citric acid accumulate most extensively following injection of malonate are primarily the heart, spleen and kidney (Busch and Potter, 1952b).

The question arises as to whether the combined action of malonate and bacteria is dependent upon living bacteria or upon the toxic effects of dead bacteria. To provide an answer, a bacterial suspension was heated at 60 C for 1 hour. It was quickly cooled and injected into mice so that each animal received 250,000 bacteria. Table 1, column 5, shows that dead bacteria, in combination with 8 injections of 20 mg malonate, were not lethal. The one fatality in the group may have been the result of an infection originating either accidentally or with organisms that survived the heat.

The results obtained when malonate was administered at hourly intervals to mice infected with graded doses of bacteria are shown in table 2. No difference in mortality was found, under the conditions of the experiments, between mice infected with 250,000 bacteria (column 3) and mice infected with 150,000 bacteria (column 4). However, mice infected with 50,000 bacteria (column 5) or with 5000 bacteria (column 6) died more slowly than mice given the larger numbers of bacteria. There is a rough proportionality noticeable, particularly during the first day or two, between number of deaths and number of bacteria, even though one of the five mice, represented in column 5, survived longer than this proportionality would suggest. Nevertheless, the mice in all groups receiving malonate died sooner, regardless of the number of bacteria, than the control mice of column 7, infected with 250,000 bacteria and injected with saline.

A similar gradation in mortality was obtained by keeping the number of bacteria constant at 250,000 and reducing the amount of malonate per injection. The results are summarized in table 3. Mice receiving injections of 15

TABLE 2.—*Survival of mice infected with graded numbers of* S. typhimurium *and injected hourly with malonate (5 mice per group).*

Time interval in hours	Total number of injections	Experimental 20 mg (½ ml) malonate hourly				Control ½ ml saline 250,000 bacteria
		250,000 bacteria	150,000 bacteria	50,000 bacteria	5000 bacteria	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
0–1	1	5	5	5	5	5
1–2	2	5	5	5	5	5
2–3	3	5	5	5	5	5
3–4	4	5	5	5	5	5
4–5	5	4	4	5	5	5
5–6	6	2	3	4	5	5
6–7	7	2	3	4	5	5
7–8	8	0	0	3	5	5
8–22	8			2	3	5
22–33	8			1	3	5
33–46	8			1	3	5
46–58	8			1	3	5
58–70	8			1	3	3
70–82	8			1	0	3
82–94	8			0		3
94–118	8					3
118–142	8					0

TABLE 3.—*Survival of mice infected with* S. typhimurium *and injected hourly with graded doses of malonate (5 mice per group).*

Time interval in hours	Total number of injections	Experimental 250,000 bacteria			Control ½ ml saline 250,000 bacteria
		20 mg malonate	15 mg malonate	10 mg malonate	
(1)	(2)	(3)	(4)	(5)	(6)
0–1	1	5	5	5	5
1–2	2	5	5	5	5
2–3	3	5	5	5	5
3–4	4	5	5	5	5
4–5	5	4	5	5	5
5–6	6	2	5	5	5
6–7	7	2	5	5	5
7–8	8	0	5	5	5
8–22	8		3	4	5
22–34	8		2	3	5
34–46	8		2	3	5
46–58	8		2	1	5
58–70	8		2	0	3
70–94	8		2		3
94–118	8		2		3
118–142	8		1		0
142–164	8		0		

mg (column 4) and 10 mg (column 5) died more slowly than those receiving 20 mg injections (column 3). Here again, a rough proportionality seemed to exist, this time between number of deaths and the dosage of the malonate, even though the last mouse given injections of 10 mg died before the last of the mice receiving 15 mg. Not included in the table are the results obtained when 40-mg doses of malonate were injected into infected and into control mice. Since all mice of both groups died within a few hours, this quantity of inhibitor is too large to be of value in these experiments.

The fact that malonate injections were started immediately after the mice were infected in all the experiments so far described was purely arbitrary and was used because of the success of the initial trials. It is of interest, therefore, to note the effect of malonate when it is given to mice for the first time about 22 hours after they were infected with 250,000 bacteria. This number of organisms produces the first death in normal mice (and hundreds have been tested) from 64 to 72 hours after they are introduced into the peritoneal cavity, except in extremely rare cases. For the first 24 hours, there are no signs of illness and the animals appear to be normal, healthy mice. An injection of 20 mg malonate, given at this time (22 hours), was followed by the death of 4 or 5 mice within the hour and the 5th mouse died in less than an hour after the second injection. These results may be seen in table 4, column 3, and should be compared with those of infected control mice given in column 5. Such a dramatic change in the course of the "infection" emphasizes the importance of the time factor in the interaction in vivo of inhibitor and pathogen.

Effect of malonate on spread of injected S. typhimurium

The fate of *S. typhimurium* injected intraperitoneally into mice assumes a new significance in light of the effects of malonate described above. There is no knowledge of the time required by the bacteria of this strain to reach the blood stream after they are injected into the peritoneal cavity nor on the duration of the septicemia after it appears. In order to obtain some information on

TABLE 4.—*Survival of mice infected with* S. typhimurium *and injected hourly the following day with malonate (5 mice per group).*

Time interval in hours	Experimental		Control	
	Total number of injections	20 mg malonate 250,000 bacteria	Total number of injections	½ ml saline 250,000 bacteria
(1)	(2)	(3)	(4)	(5)
0–1	0	5	1	5
1–2	0	5	2	5
2–3	0	5	3	5
3–4	0	5	4	5
4–5	0	5	5	5
5–6	0	5	6	5
6–7	0	5	7	5
7–8	0	5	8	5
8–22	0	5	8	5
22–23	1	1	8	5
23–24	2	0	8	5
24–48			8	5
48–72			8	3
72–96			8	3
96–120			8	1
120–144			8	0

these questions, plates of SS agar (Difco), a differential nutrient medium on which members of the genera *Salmonella* and *Shigella* give colonies with a characteristic color, were inoculated with tail blood of 10 mice infected with 250.000 cells of *S. typhimurium*. Five of the mice were given hourly injections of saline and five were given hourly injections of 20 mg of malonate, beginning immediately after infection and continuing for a total of 8 injections. Blood was obtained every hour by amputating the tip of the tail, pressing out a small drop of blood, touching it to the surface of the agar, and smearing it over a sector of the plate with an inoculating loop. This was done with each mouse for a total of 12 hours, with the results shown in table 5. The larger the number entered in the table, the greater the amount of bacterial growth, but this is only approximately quantitative since the volume of blood used as inoculum was not measured. Nevertheless, the data show that bacteria reach the blood stream within a period of approximately 3 to 5 hours in control mice, and in approximately the same time in mice receiving malonate. The bacteria leave the blood stream of the control mice 10 to 12 hours after infection, except in the one mouse that died within about 24 hours—an unusually short time for death in control mice. The major difference that appears in mice treated with malonate, compared to controls, is the very heavy and early septicemia prior to and at the time of death, suggesting that a relationship exists between the inhibitor and the severity of the blood stream infection. Additional experiments along these lines are now in progress.

DISCUSSION

The only certain fact emerging from these experiments is that the survival time of mice infected with *S. typhimurium* is reduced by repeated injections of sublethal amounts of sodium malonate. This is seen when the malonate injections are begun immediately after the animals are infected and when survival time is compared to that of control mice similarly infected and given comparable injections of normal saline. The amount by which the survival time is reduced in mice infected with a given number of bacteria is dependent upon the quantity of malonate given per injection. Conversely, for a fixed amount of malonate, the survival time is decreased by increasing the number of bacteria up to a point. However, the same survival time was obtained with mice given either 150,000 bacteria or 250,000 bacteria (table 2).

TABLE 5.—*Relative amount of bacterial growth on SS agar (Difco) from blood of mice infected at time zero with 250,000 cells of* S. typhimurium *and then injected at hourly intervals for 8 injections with either 0.5 ml saline or 20 mg malonate.*

Hours	Saline					Malonate				
	1	2	3	4	5	1	2	3	4	5
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	1	0	0	0	0	0	0
3	1	1	0	1	1	0	1	1	1	1
4	1	1	0	2	0	1	2	3	2	2
5	2	1	1	2	2	2	2	2	2	2
6	2	3	2	2	2	2	3	3	3	3
7	2	2	2	2	2	3	3	3	Dead	3
8	2	2	2	3	3	3	3	4		Dead
9	2	2	2	2	3	Dead	4	Dead		
10	2	2	0	2	2		4			
11	0	0	0	1	2		4			
12	0	0	0	0	2		4			
25	0	0	0	3	Dead		Dead			

Various explanations are possible for these observations, but the evidence available at this time permits little choice between them.

The strongest temptation is to interpret the results on the basis of an interference with the tricarboxylic acid cycle, one reaction of which malonate is known to block (Krebs et al, 1952). However, since it may also inhibit reactions which are not part of the cycle (Pardee and Potter, 1949), its effect need not be exclusively attributed to interference with that particular pathway. In any event, the reduction in survival time of infected mice by malonate makes it apparent that the ultimate effect is against the mouse and not the bacteria. That is, bacterial metabolism cannot be significantly inhibited since, as seen in the data of table 5, there is an apparent increase in number of organisms infecting the blood stream of mice receiving malonate. This increase in number of bacteria may be the result of an impaired cellular defense due to the unfavorable effect of malonate on phagocytes. This is subject to experimental test, but since mice that have been infected for about 22 hours die within an hour or two after malonate is injected (table 4), it is unlikely that an increase in bacterial population is the significant factor responsible for the death of mice under the particular circumstances. It is also unlikely that malonate acts to select or to induce changes in the virulence of bacteria in some manner similar to the action of threonine described by Page et al (1951). Malonate produces its effect within hours and threonine requires days.

If it is postulated that survival time of infected mice is reduced by malonate because of a partial blockade of the Krebs cycle—and whether this proves to be the case or not it must be remembered that malonate does seem to alter the normal functioning of the cycle under in vivo conditions (cf. Busch and Potter, 1952a),—the underlying basis for a link between an infection and the normal functioning of the metabolic machinery of the host remains obscure. The fact that inhibitors of the citric acid cycle other than malonate and intermediates of the cycle known to accumulate in the presence of the inhibitors are able, like malonate, to reduce the survival time of infected mice, as seen in the paper which follows (Berry and Mitchell, 1953b), strengthens the implication that some linkage does exist between host metabolism and response to pathogenic bacteria. Only additional experiments can provide the basis for a better understanding of this phenomenon.

SUMMARY

The survival time of mice infected intraperitoneally with a standardized suspension of *Salmonella typhimurium* varies between 64 and 84 hours, and it remains unchanged when 0.5 ml of normal saline is injected at hourly intervals for a total of 8 injections starting immediately after the mice are inoculated with the bacteria. Survival time is reduced to 8 to 24 hours in mice similarly infected but given 8 injections of 20 mg of malonate. Survival time increases either with a decrease in the amount of malonate (between 20 and 10 mg) given per injection or with a decrease in the number of bacteria (between 150,000 and 5000) with which the mice are infected, but the maximum survival time of these mice is less than the minimum survival time of control mice.

Injections of malonate into mice inoculated with 250,000 killed bacteria are without lethal effect. However, when the first malonate injection is given 22 hours after the mice were infected, 4 of

5 mice died within an hour and the 5th mouse died after the second injection of malonate.

Blood from the tail of mice infected intraperitoneally with *S. typhimurium* and injected at hourly intervals with either 20 mg malonate or with an equivalent volume of normal saline was cultured on SS agar. The cultures were positive for salmonella (or shigella) 2 to 5 hours after infection in both groups of mice, but the cultures from mice receiving the malonate were heavier than those from mice receiving saline. Qualitatively, it appears that the malonate injections are accompanied by an overwhelming septicemia, which never permits a negative blood culture to develop as it does in control mice 10 to 12 hours after infection.

Since the work of other investigators has shown malonate capable of altering in vivo the normal functioning of the tricarboxylic acid cycle, these results are tentatively attributed to some as yet unexplained linkage between this cycle and susceptibility to infection.

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THE RELATION OF THE TRICARBOXYLIC ACID CYCLE TO BACTERIAL INFECTION

II. THE EFFECT OF FLUOROACETATE, ARSENITE, CITRATE, AND SUCCINATE ON *SALMONELLA TYPHIMURIUM* INFECTIONS IN MICE

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In the first paper of this series (Berry and Mitchell, 1953), it was established that injections of sublethal amounts of sodium malonate in mice infected intraperitoneally with *Salmonella typhimurium* reduce survival time from a minimum of approximately 65 hours to a maximum of about 24 hours. This is observed when the malonate injections are started immediately after the mice are infected and are repeated at hourly intervals for a total of 8 injections. Busch and Potter (1952a and b) observed that malonate injections in rats increase the concentration of succinate in blood and organs, followed by an increase in the concentration of succinate and citrate excreted in the urine. This confirmed an earlier report by Krebs et al (1938) for rats and rabbits, but these workers also found greater quantities of α-ketoglutaric acid in the urine of animals given sublethal amounts of malonate.

If it is assumed that malonate increases in vivo the concentration of succinate and citrate because it competitively inhibits (Krebs et al, 1952) succinic dehydrogenase and possibly other enzymes requiring free magnesium ions (Pardee and Potter, 1949), then the tricarboxylic acid cycle in these animals is partially blocked. It must also be assumed that the synergism in mice between malonate and *S. typhimurium* responsible for the reduction in survival time is due to one of two modes of action. Either the malonate exerts its effect directly as malonate or indirectly through the intermediates made available by its action. In view of the latter possibility, experiments were undertaken to determine the extent to which other poisons known to block enzymes in the tricarboxylic acid cycle or to which intermediates of the cycle known to accumulate in malonate poisoning were able to duplicate the role played by malonate in reducing the survival time of mice infected with *S. typhimurium*. This report, therefore, presents the results obtained when the inhibitors, sodium fluoroacetate or sodium arsenite, or the intermediates, sodium citrate or sodium succinate, were substituted for malonate.

Fluoroacetate has come into prominence as an important Krebs cycle inhibitor since the end of World War II. Injections of quantities ranging from 3 to 10 mg per kg of body weight in mice lead to an accumulation of citric acid in the different organs of the body, particularly in the heart, kidney and spleen (Buffa and Peters, 1950). A dose of 10 mg per kg is cited by some authors (Ward and Spencer, 1947) as the LD_{50} dose for mice, and other workers find 10 to 20% mortality with 3 to 4 mg per kg (Mogabgab and Horsfall, 1952). In the present report 8 mg per kg give 25%

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mortality with the mice used in these studies. It must be inferred from these findings that strains of mice vary in their sensitivity to this poison. The isolation by Peters, Wakelin and Buffa (1952) of a fluorine containing tricarboxylic acid makes it seem likely that fluoroacetate becomes incorporated into the Krebs cycle by undergoing some of the reactions postulated for acetate. It is the fluoro-tricarboxylic acid which acts as a competitive inhibitor for the enzyme aconitase, according to Peters and Wilson (1952), and a blockage of this enzyme permits citric acid to accumulate. The block from a single dose of fluoroacetate may last from 4 to 8 hours (Ackermann, 1951; Lindenbaum et al, 1951) while daily injections for a total of 18 days fail to alter the type or magnitude of the response elicited (Lindenbaum et al, 1951). In addition to the block of the Krebs cycle, fluoroacetate has been shown to lower the oxidation of fatty acids (Kalnitsky and Barron, 1948) and to reduce the conent of certain amino acids in the organs of rats (Awapara, 1952). The metabolism of both fats and amino acids might well be altered by fluoroacetate because of its action on the Krebs cycle.

Early work with arsenite as a metabolic poison was carried out by Onaka (1911) in Warburg's laboratory and later by Dresel (1926 and 1928). They established the fact that it was a powerful respiratory inhibitor, but it was not until 1933 that Krebs attributed its action to a blocking of α-ketoglutaric oxidation,—the step responsible for the formation of succinic acid. Arsenite, therefore, blocks the reaction that is thought to precede the reaction inhibited by malonate. This relationship is of interest in attempting to analyze the results previously described for malonate (Berry and Mitchell, 1953), but Peters, Sinclair and Thompson (1946) present good reason to believe that arsenite has additional action. In animals poisoned with arsenite there is an elevated blood pyruvate concentration due apparently to the fact that the pyruvate oxidase system is the most sensitive of the oxidases to trivalent arsenical poisoning and the pyruvate dehydrogenase the most sensitive of the dehydrogenases studied. Additional investigations with this inhibitor are needed.

Sodium citrate and sodium succinate were included in this work because both compounds are known experimentally to accumulate in vivo in the presence of malonate (Busch and Potter, 1952a and b; Potter and Busch, 1950) and the former in the presence of fluoroacetate (Lindenbaum et al, 1951; Potter et al, 1951). Other tricarboxylic and dicarboxylic acids may also accumulate in the presence of these inhibitors but the evidence is not available. Citrate is toxic when given in sufficiently large doses (Gruber and Halbeisen, 1948), but the quantity required exceeds the amount used in this work by a factor of approximately five. Its exact place in the Krebs cycle remains in doubt (Baldwin, 1952), but even if it is an equilibrium compound not directly on the main pathway, it is useful experimentally as an assay compound. Succinate, on the other hand, seems beyond question to be directly involved in the reactions of the cycle. It is toxic only when given in doses several times greater than the one used in this study, according to our own experiences, but no toxicity data have been found in the literature.

MATERIALS AND METHODS

Inhibitors

Sodium monofluoroacetate, kindly supplied by Monsanto Chemical Company, was used without further purification. According to the manufacturer's assay, the material was 90% pure, with 10% inert ingredient. Sterile normal saline was

used as the solvent and a fresh solution was prepared for each day's injections. The final solution was diluted so as to contain the desired quantity in about 0.5 ml. The mice were injected with one of the following doses: 1, 2, 3, 4, 5, or 8 mg per kg of mouse. In the first series of experiments with fluoroacetate, mice to be injected were selected by weight so that all were within 1.0 g of 20 g and all received the inhibitor in the same volume of fluid.

Sodium arsenite (Merck reagent) was dissolved fresh for each experiment in sterile normal saline. Three dosage levels were studied, 0.5, 0.25, and 0.125 mg per mouse per injection. The concentrations were prepared in such a way that the desired quantity of arsenite was contained in 0.5 ml of solution.

Metabolites

Trisodium citrate (Merck reagent) was dissolved in sterile normal saline so that the final concentration gave the desired amount in 0.5 ml of solution. Three dosage levels were used: 2, 5, and 10 mg per mouse per injection.

Disodium succinate (Merck) was prepared fresh for each experiment and it was dissolved in sterile normal saline. Only one dosage level was used, namely, 20 mg per injection per mouse.

Bacterial suspension

All mice were infected intraperitoneally with a suspension containing approximately 250,000 cells of *S. typhimurium* in a volume of 0.5 ml. The suspension was prepared according to the procedure described in the preceding paper (Berry and Mitchell, 1953).

General

Young adult female mice of the CF-1 strain were used for some experiments and young adult male mice of the Detwiler strain were used for the remaining experiments. The particular strain employed is designated in the results. Injections of inhibitors and intermediates were given intraperitoneally. For further details, see Berry and Mitchell (1953).

RESULTS

1. *Experiments with sodium fluoroacetate*

Female mice of the CF-1 strain were infected in the usual way with *S. typhimurium* and the 1st injection of fluoroacetate was given immediately thereafter. Twenty-two hours later, the 2nd injection was given, followed 5 hours later by the 3rd. Five different doses were used with the fluoroacetate solutions prepared in such a way that all mice received approximately the same volume of fluid. Dosages of 5, 4, 3, 2, or 1 mg of fluoroacetate per kg of mouse per injection gave the results shown in table 1. All mice that received both bacteria and fluoroacetate (columns 3, 5, 7, 9, and 11) died a few hours sooner than the infected control mice (column 13), but the differences are too small to be significant with the number of animals employed. There is no apparent proportionality between the dosage of inhibitor and the time required for 100% mortality. Moreover, mice receiving inhibitor only (columns 4, 6, 8, 10, and 12) died almost as quickly as the infected mice. The cause

TABLE 1.—*Survival of mice infected with* S. typhimurium *and injected at intervals with graded doses of sodium fluoroacetate (5 mice per group).*

Time interval in hours	Total number of injections	Experimental										Control ½ ml saline
		5 mg/kg		4 mg/kg		3 mg/kg		2 mg/kg		1 mg/kg		
		250 T bacteria	No bacteria	250 T bacteria	No bacteria	250 T bacteria	No bacteria	250 T bacteria	No bacteria	250 T bacteria	No bacteria	250,000 bacteria
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
0–1	1	5	5	5	5	5	5	5	5	5	5	5
1–2	1	5	5	5	5	5	5	5	5	5	5	5
2–10	1	5	5	5	5	5	5	5	5	5	5	5
10–22	1	5	5	5	5	4	5	4	5	5	5	5
22–34	3	4	5	5	5	4	5	4	5	5	5	5
34–46	3	4	4	4	5	4	5	4	5	5	5	5
46–58	3	4	4	3	5	4	5	4	5	5	5	5
58–70	3	3	3	1	4	3	5	4	5	4	5	3
70–94	3	1	3	0	2	1	5	3	5	0	5	3
94–118	3	1	3		2	1	5	3	5		5	3
118–142	3	0	2		1	0	3	0	4		3	0
142–166	3		1		1		2		4		2	

of death in these mice is not certain, but it is assumed that most died of accidental infections. Confirming this assumption are the results summarized in table 2 (see below).

In the 2nd experiment with fluoroacetate, the mice were given only 2 injections, the 1st immediately after the bacteria were administered and the 2nd 6 hours later. Two different dosages were employed. One was larger than any used in the 1st experiment, 8 mg per kg, and the 2nd, 5 mg per kg, was included as a check on the 1st experiment. The results are summarized in table 2. Infected mice which received the larger dose of inhibitor (8 mg per kg) were all dead (column 5) before any of the infected control mice died (column 7). Infected mice given 5 mg of fluoroacetate per kg of mouse (column 3) died slightly sooner than the control mice but, like the results in table 1, the difference is equivocal. It is important to note, moreover, that uninfected mice injected with either the 5 mg per kg doses or the 8 mg per kg doses survived, except for one animal in the latter group. This is seen in columns 4 and 6 of table 2 and substantiates the assumption made concerning the deaths of comparable mice in the 1st experiment. The cause of the one death listed in column 6 is unknown, but this quantity of fluoroacetate is very close to the reported LD_{50} level (Ward and Spencer, 1947), and with some strains of mice it exceeds the amount certain to produce some deaths (Mogabgab and Horsfall, 1952). On the strength of the data of table 2, it is permissible to conclude that fluoroacetate in a concentration close to the lethal level acts synergistically with *S. typhimurium* to produce 100% mortality in a small group of mice before any animals in a group receiving the bacteria alone succumb.

2. Experiments with sodium arsenite

Table 3 summarizes the results obtained when Detwiler mice were infected with 250,000 cells of *S. typhimurium* followed immediately by (a) 8 hourly injections of saline (columns 12 and 13), (b) a single injection of 0.5 mg of arsenite (column 3), (c) 2 injections of 0.25 mg arsenite, the 2nd given 2 hours after the 1st (column 6), and (d) 3 injections of 0.125 mg of arsenite, the 2nd given 1 hour after the 1st and the 3rd 1½ hours after the 2nd (column 9). The 2 largest doses (0.5 and 0.25 mg) were lethal without bacteria (columns 4 and 7, respectively), and hence no significant differences could be expected between the survival of infected and uninfected animals administered these quantities of arsenite. However, when 0.125 mg arsenite was used, all the infected mice died (column 9) before any of the

TABLE 2.—*Survival of mice infected with* S. typhimurium *and injected at intervals with sodium fluoroacetate (FA) (6 mice per experimental group, 11 mice control group).*

Time interval in hours	Total number of injections	Experimental				Control ½ ml saline 250,000 bacteria
		5 mg FA/per kg		8 mg FA/per kg		
		250,000 bacteria	No bacteria	250,000 bacteria	No bacteria	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
0–6	1	6	6	6	6	11
6–15	2	6	6	6	6	11
15–39	2	4	6	5	5	11
39–47	2	4	6	0	5	11
47–63	2	3	6		5	7
63–87	2	3	6		5	6
87–111	2	2	6		5	5
111–135	2	0	6		5	3
135–159	2		6		5	2

TABLE 3.—*Survival of mice infected with* S. typhimurium *and injected at intervals with graded doses of sodium arsenite (SA) (5 mice per group).*

Time interval in hours	Experimental									Control		
	0.5 mg SA			0.25 mg SA			0.125 mg SA			Total injections	½ ml saline 250,000 bacteria	
	Total injections	250 T bacteria	No bacteria	Total injections	250 T bacteria	No bacteria	Total injections	250 T bacteria	No bacteria		Group 1	Group 2
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
0–1	1	5	5	1	5	5	1	5	5	1	5	5
1–2	1	3	1	1	4	4	2	5	5	2	5	5
2–3	1	3	0	2	3	3	2	5	5	3	5	5
3–4	1	2		2	0	1	3	3	5	4	5	5
4–5	1	0		2		1	3	2	4	5	5	5
5–12				2		1	3	2	4	8	5	5
12–24				2		1	3	1	4	8	5	5
24–48				2		1	3	0	4	8	5	5
48–72				2		1	3		4	8	4	4
72–96				2		1	3		4	8	2	2
96–120				2		0	3		4	8	1	1
120–144							3		4	8	0	0

infected control mice (columns 12 and 13). One of the 5 mice given only the 3 injections of 0.125 mg of inhibitor died (column 10). The death of this animal may have been due to the poison or to an injury incurred during the injections. The same amount of arsenite administered in the same way to 2 other groups, each with 5 control mice, failed to kill any of them, which indicates that such quantities of arsenite are not likely to produce as much as 10% mortality. It is obvious, therefore, that the arsenite and the bacteria combine to reduce the survival time of mice in much the same way and to approximately the same extent as fluoroacetate and bacteria.

3. Experiments with sodium citrate

As explained in the introduction, these experiments were undertaken because citrate is known to accumulate in animals poisoned with either malonate (Bush and Potter, 1953b) or fluoroacetate (Buffa and Peters, 1950). Accordingly, CF-1 mice infected with 250,000 cells of *S. typhimurium* were given 8 hourly injections starting immediately after infection with one of three quantities of citrate, 10, 5, or 2 mg. The results are shown in table 4. Infected mice given citrate (columns 3, 4, and 5) died before the infected control mice injected with saline (column 6). Moreover, the survival time of the mice was inversely proportional to the quantity of citrate administered. The complete survival of control mice injected 8 times with 10 mg of citrate is not shown in table 4, but it appears in column 6 of table 5. The LD_{50} dose for trisodium citrate is about 50 mg when it is given in a single intraperitoneal injection (Gruber and Halbeisen, 1948).

In order to determine the effect of the time at which the series of citrate injections was given relative to the time of infection, the following experiment with Detwiler mice was undertaken. Table 5 shows the mortality of mice infected with 250,000 cells of *S. typhimurium* (a) followed by 8 hourly injections of 10 mg citrate (column 3), (b) after 4 hourly injections of 10 mg citrate followed by 4 hourly injections of the same amount of citrate (column 4), and (c) after 8 hourly injections of 10 mg citrate (column 5). Under the conditions of the experiment, administering the bacteria after either half or all of the citrate injections were complete yielded mortality data essentially like those obtained when no citrate at all was in-

TABLE 4.—*Survival of mice infected with* S. typhimurium *and injected hourly with sodium citrate (5 mice per group).*

Time interval in (hours)	Total injections	Experimental			Control ½ ml saline 250,000 bacteria
		10 mg citrate 250,000 bacteria	5 mg citrate 250,000 bacteria	2 mg citrate 250,000 bacteria	
(1)	(2)	(3)	(4)	(5)	(6)
0–1	1	5	5	5	5
1–2	2	5	5	5	5
2–3	3	4	5	5	5
3–4	4	4	5	5	5
4–5	5	3	5	5	5
5–6	6	1	5	5	5
6–7	7	0	5	5	5
7–8	8		5	5	5
8–22	8		0	3	5
22–34	8			3	5
34–46	8			3	5
46–58	8			3	5
58–70	8			1	3
70–82	8			0	3
82–94	8				3
94–106	8				3
106–118	8				3
118–130	8				1
130–142	8				0

jected. This is apparent when the results in columns 4 and 5 are compared with those of columns 3 and 7. At the same time, all mice given the citrate injections after infection died before any of the infected control mice. Whatever the mechanism by means of which citrate reduces the survival time of infected mice, the results of this experiment prove that it is short-lived. If the citrate injections weaken the mouse so as to make it more susceptible to infection, then the mouse recovers very quickly. If, on the other hand, the citrate acts on the bacteria, rendering them, in some way, more virulent, then these results imply that the bacteria are able to respond only after they have been in the mouse for several hours. This last concept is strongly supported by a relation-

TABLE 5.—*Survival of mice infected with* S. typhimurium *at different times with respect to hourly injections with citrate (5 mice per group).*

Time interval in hours	Total number of injections	Experimental				Control ½ ml saline 250,000 bacteria
		10 mg sodium citrate hourly				
		250,000 bacteria at arrow	250,000 bacteria at arrow	250,000 bacteria at arrow	No bacteria	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
		→				
0–1	1	5	5	5	5	5
1–2	2	5	5	5	5	5
2–3	3	5	5	5	5	5
3–4	4	5	5	5	5	5
			→			
4–5	5	5	5	5	5	5
5–6	6	5	5	5	5	5
6–7	7	5	5	5	5	5
7–8	8	5	5	5	5	5
				→		
8–24	8	2	5	5	5	5
24–36	8	2	5	5	5	5
36–48	8	1	5	5	5	5
48–60	8	1	5	5	5	5
60–72	8	0	3	3	5	4
72–96	8		2	0	5	2
96–120	8		1		5	1
120–144	8		1		5	0
144–168	8		0		5	

ship known to exist with malonate (Berry and Mitchell, 1953). Six to eight injections of malonate are required to kill mice immediately after they are infected but one or two injections kill when they are given 22 hours after infection. Additional studies along these lines are indicated.

Attention is directed to the difference in survival time of the infected mice given 8 injections of 10 mg of citrate, as shown in table 4, column 3, compared to the mice given identical treatment as seen in table 5, column 3. The mice in the 1st group were all dead after 7 hours while the last mouse in the 2nd group did not die for about 72 hours. The mice were of different strains, different ages, and different sexes, any one of which might account for the difference in response.

4. Experiments with sodium succinate

Injections at hourly intervals of 20 mg of succinate, beginning immediately after Detwiler mice had been infected with 250,000 cells of *S. typhimurium*, resulted in the death of 5 out of 5 mice 1 day prior to the death of any of the mice similarly infected but injected with saline. These results may be seen in table 6 when column 3 is compared with column 5. All mice receiving only the succinate injections survived, as shown in column 4. Thus succinate, like citrate, reduces the survival time of mice infected with the mouse typhoid organism.

TABLE 6.—*Survival of mice infected with* S. typhimurium *and injected hourly with sodium succinate (5 mice per group).*

Time interval in hours	Total number of injections	Experimental 20 mg succinate		Control ½ ml saline
		250,000 bacteria	No bacteria	250,000 bacteria
(1)	(2)	(3)	(4)	(5)
0–1	1	5	5	5
1–2	2	5	5	5
2–3	3	5	5	5
3–4	4	5	5	5
4–5	5	5	5	5
5–6	6	5	5	5
6–7	7	5	5	5
7–8	8	5	5	5
8–24	8	1	5	5
24–36	8	1	5	5
36–48	8	0	5	5
48–60	8		5	5
60–72	8		5	4
72–96	8		5	2
96–120	8		5	1
120–144	8		5	0

DISCUSSION

The results of the experiments described above establish the fact that injections of appropriate amounts of fluoroacetate, arsenite, citrate or succinate reduce the survival time of mice infected with *S. typhimurium*. Malonate also has a similar action (Berry and Mitchell, 1953). Since all five compounds produce a common effect, the cause must be either nonspecific and different for each substance or it must be common to all. Each of the two possibilities will now be considered.

If it is assumed, first, that the cause is nonspecific, then it is conceivable that mice reduced to marginal survival by the toxic action of one of the compounds are unable to withstand the additional stress of bacterial infection. Implied in this interpretation is the ability of two unrelated sublethal effects to combine into one terminating fatally, a relationship known to exist both pharmacologically and physiologically. The quantity of fluoroacetate (8 mg per kg) or of arsenite (0.125 mg per injection) or of malonate (20 mg per injection) (Berry and Mitchell, 1953) necessary to produce an obvious reduction in survival time is, for each substance, toxic to control mice. The toxicity manifests itself in the labored breathing, the ruffled fur, the absence of activity, and the reclining posture of the animals. Fluoroacetate alone killed 1 of 6 control mice, table 2, but subsequent tests have expanded these figures to read 4 killed out of 16, a 25 % mortality. Arsenite

alone, as previously mentioned, killed only 1 of 15 mice, but this one death may not have been due to the arsenite. Malonate, on the other hand, when given in the quantity designated above, has killed no mice out of 30, while citrate and succinate fail to elicit toxicity symptoms. Nevertheless, both intermediates are just about as active as the more toxic compounds in reducing the survival time of infected mice. Moreover, as table 2 shows, 5 of 6 infected mice given 2 injections of 8 mg per kg of fluoroacetate died between the 39th and 47th hours, about 12 hours after the toxic manifestations of the inhibitor had disappeared, insofar as this can be judged by the in vivo accumulation of citric acid (Lindenbaum et al, 1951) and on the basis of the behavior of the mice themselves. The inhibitor and the bacteria act sequentially, rather than simultaneously, as a summation of sublethal effects would predict. If the 5 compounds act nonspecifically to produce a common effect, they must act not by virtue of toxicity alone but rather by some means yet to be determined. This does not exclude the possibility that the bacteria themselves are toxic by virtue of enzyme inhibition, which conceivably would then add to the inhibition of one of the poisons.

The alternative interpretation suggests that survival time is reduced because all of the five compounds tested produce a similar effect within the infected mouse. The inhibitors block the citric acid cycle and intermediates accumulate in vivo when the cycle is blocked. This affords then a basis for common action, particularly if it is assumed that the ultimate cause is dependent upon the accumulation of one, or possibly several, intermediates. With inhibitors alone, it would be reasonable to postulate a deficiency of some intermediate as the cause for death in an infected mouse, but there is no obvious way in which injections of either citrate or succinate would lead to a deficiency of any intermediate in the cycle. If, on the other hand, some intermediate such as citric acid reached concentrations in vivo higher than those normally present, it would make available to the bacteria a source of food for which they otherwise might have to compete. In this way the bacteria would be favored so that the infection could progress more rapidly. Using this interpretation, it is possible to explain why the course of infection remains unaltered when the citrate injections were given prior to the introduction of the bacteria, as seen in table 5. If the added citrate were metabolized by the mouse at such a rate that no appreciable accumulation occurred, then when the bacteria are injected they would be unable to benefit from the previously administered citrate.

While this interpretation is speculative to a large degree, it nevertheless helps to explain experiments otherwise difficult to interpret, and, even more important, it permits predictions which are subject to experimental verification. The greater susceptibility to *S. typhimurium* infection in mice subjected to the stress of the hypoxia of a simulated high altitude (Berry and Mitchell, 1951) might be due to the accumulation of certain intermediates favorable to the bacteria. At least such mice should be carefully checked for metabolic disturbances of this type. Moreover, if bacterial counts were made in mice given injections of inhibitor or intermediate, and if these counts were compared to those from control mice, it would indicate whether or not the injections permitted a more rapid increase in the bacterial population within the mouse. Preliminary experiments indicate that this is the case to a very significant extent. This interpretation

also predicts that mice infected with different species of pathogenic bacteria might respond quite differently to injections of the compounds used in these studies. Again, preliminary results show that differences do appear. If additional work substantiates these findings then it is apparent that a better understanding of host-parasite relationships can be obtained by the use of metabolic inhibitors and intermediates.

SUMMARY

Six mice infected intraperitoneally with a standardized suspension of *Salmonella typhimurium* followed by two injections of sodium fluoroacetate died within 47 hours. Eleven control mice similarly infected but given injections of normal saline in place of the fluoroacetate suffered their first casualty after about 63 hours and their last after more than 6½ days. One of the six mice given only fluoroacetate died, but subsequent tests make it appear that about 25% of the strain of mice used in these studies die from this quantity of fluoroacetate. Mice similarly infected but given 3 injections of sodium arsenite died before any of the infected control mice. This quantity of arsenite alone killed 1 of 5 mice, but none of 10 mice subsequently tested died.

Both fluoroacetate and arsenite are known to be inhibitors of enzymes functioning in the tricarboxylic acid cycle and intermediates of that cycle are known from the work of others to accumulate in vivo when they are administered. Thus, mice infected with *S. typhimurium* followed by 8 injections, at hourly intervals starting immediately after the bacteria were introduced, of either sodium citrate or sodium succinate died before any of the control mice similarly infected and given 8 injections of normal saline. The two inhibitors and the two intermediates used in these experiments have the common property of reducing the survival time of mice infected with *S. typhimurium*. However, when the 8 citrate injections were given before the mice were infected or when half of the injections were given before infection, the survival time of the mice was not significantly different from that of infected control mice. This suggests that the bacteria must be in the mice if the citrate is to reduce survival time.

These results are tentatively offered as evidence for a linkage between host metabolism and infection. Imbalance in the tricarboxylic acid cycle of the host appears to increase susceptibility to the pathogen used in these studies.

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THE ACTION OF MUCIN IN PROMOTING INFECTIONS: THE EFFECT OF A PREVIOUS INJECTION OF MUCIN AND OF α-TOCOPHEROL IN PEANUT OIL

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Elberg and Gale (1949) reported that intraperitoneal injection of α-tocopherol dissolved in peanut oil or of hog gastric mucin protected animals from a subsequent infection with *Salmonella typhi* in hog gastric mucin. The time interval between the two injections was 48 hours. These and later observations (Gale and Elberg, 1952) were put forward as support for a theory on the mechanism by which hog gastric mucin promotes infections. This theory was based on changes in capillary permeability thought to be induced by α-tocopherol and by mucin. While it is possible that changes in capillary permeability may affect the course of an infection promoted by mucin, examination of the figures given in table 1 (Elberg and Gale, 1949) suggested that a nonspecific immunity might be an adequate explanation of the results using α-tocopherol in peanut oil.

It was therefore decided to repeat the experiments with both α-tocopherol in peanut oil and with hog gastric mucin, using large batches of mice. This paper describes the results of the investigation. In addition, the repercussion is stressed of recent chemical work identifying the factors involved in the infection-promoting effect of mucin on investigations into its mode of action.

MATERIALS AND METHODS

Mucin.—This was the "standard" batch of Wilson's Granular hog gastric mucin described by Smith (1950a).

Preparation and sterilization of the mucin suspensions.—Full details are given by Smith (1950a).

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α-Tocopherol.—Three different batches of α-tocopherol were supplied by Roche Products Ltd., Welwyn Garden City, London, England. The requisite quantity was dissolved in 0.2 ml sterile peanut oil for injection.

S. typhi.—The virulent strain "Ty_2" was used. This strain and the methods of subculture and counting are described by Smith (1950a). The LD_{50} for this strain when injected in tryptic meat broth is approximately 5×10^8.

Mice.—Eighteen- to twenty-two-gram white mice were used.

Preliminary injections.—These were made either subcutaneously or intraperitoneally.

Solutions for the challenge injection.—The same procedure as that described by Smith (1950a) was adopted. Five-tenths ml of a mixture of 9 parts of the mucin suspension (or tryptic meat broth in the case of the controls) and 1 part of a tryptic meat broth dilution of *S. typhi* which contained the requisite number of organisms was injected. The number of organisms used with mucin was approximately 1000 times less than the normal LD_{50}, i.e. 5×10^3. A viable count was made on each suspension used.

Period of observations.—Observations were made for 3 days after the challenge injection.

RESULTS

Table 1 summarizes the results obtained when a preliminary injection of α-tocopherol in peanut oil is given. Preliminary subcutaneous injections have no observable action, but an effect is apparent in animals given a preliminary intraperitoneal injection. Our colleague, Mr. S. Peto, has calculated from a combination of the three tests that there is a significant drop in the death rate of the mice previously injected with α-tocopherol in peanut oil, compared with the control group; but there is also a significant drop produced by the preliminary injection of peanut oil alone. There is no significant difference between the death rates in groups of mice injected with different amounts of α-tocopherol, or with peanut oil alone.

TABLE 1.—*Effect of an injection of α-tocopherol in peanut oil on a subsequent mucin infection with* S. typhi.

Preliminary injection (0.2 ml)	Second injection (0.5 ml intraperitoneally after an interval of 48 hours)	Deaths (numerator) in batches (denominator) of mice after preliminary injection of the batches of α-tocopherol by the route (intraperitoneally—I.P., subcutaneously—S.C.) stated					
		Batch 1		Batch* 2		Batch 3	
		I.P.	S.C.	I.P.	S.C.	I.P.	S.C.
10 mg α-tocopherol in peanut oil	5×10⁸ *S. typhi* in mucin†	5/10	5/10	14/40	31/40	15/19	17/20
1 mg α-tocopherol in peanut oil	5×10⁸ *S. typhi* in mucin†	6/10	7/10	13/40	31/40	10/20	17/20
0.1 mg α-tocopherol in peanut oil	5×10⁸ *S. typhi* in mucin†	9/10	7/10	20/40	32/39	6/20	17/20
Saline	5×10⁸ *S. typhi* in mucin†	9/10	7/10	31/40	31/40	17/20	16/20
Peanut oil alone	5×10⁸ *S. typhi* in mucin†	9/10	9/10	16/40	30/40	7/20	18/20
10 mg α-tocopherol in peanut oil	Mucin† only			0/20	0/10	0/10	1/10
Saline	Mucin† only	1/10	0/10	0/20	0/20	1/10	1/10
Saline	5×10⁸ *S. typhi* in tryptic meat broth			1/20	0/10	0/10	0/10
Saline	5×10⁸ *S. typhi* in tryptic meat broth	5/10	5/10	17/20	16/20	6/10	10/10

The strain of *S. typhi* and the sample of mucin used, the preparation and sterilization of mucin suspensions, and the final preparation of injection solutions are described in detail by Smith (1950a).
* For the sake of brevity these figures are a combination of those of two identical experiments.
† Concentration of mucin used was 1.75% w/v for batch 1, 1.5% w/v for batches 2 and 3.

Table 2 summarizes the results obtained when a preliminary injection of mucin has been given. Here preliminary subcutaneous or intraperitoneal injection of mucin lowers the subsequent death rate. Significant differences from the control are produced by preliminary intraperitoneal injection of mucin 24 to 48 hours previous to the challenge. Preliminary subcutaneous injection, 24 hours before the challenge dose, produces a small difference from the control group; this difference becomes significant when the interval between the injections is 48 hours.

DISCUSSION

Elberg and Gale (1949) conclude from their experiments that α-tocopherol antagonizes the effect of mucin in promoting bacterial infections. They attribute this action to a specific effect of α-tocopherol on capillary permeability, and on this assumption base certain conclusions about the mode of action of mucin. These arguments are elaborated in another paper (Gale and Elberg, 1952), in which further experiments are based partly on their original findings with α-tocopherol. In their basic experiment the α-tocopherol was dissolved in peanut oil, and of the three mice in the peanut oil control group, one survived the subsequent challenge with mucin and *S. typhi*. The work now reported, in which much larger groups of animals were used, makes it clear that peanut oil does afford protection,

TABLE 2.—*Interference by an injection of mucin with a subsequent mucin infection with* S. typhi.

Percent concentration of preliminary mucin injection (0.5 ml)	Challenge, intraperitoneal injection (0.5 ml)		Deaths (numerator) in batches (denominator) of mice after the preliminary injection by the route and after the time interval stated			
	Percent mucin	*S. typhi*	Intraperitoneally		Subcutaneously	
			24 hr.	48 hr.	24 hr.	48 hr.
Exp. 1*						
2.0	1.75	5×10⁸	4/20	1/20	8/20	3/20
1.5	1.75	5×10⁸	1/20	1/20	6/20	3/20
1.0	1.75	5×10⁸	4/20	3/20	9/20	4/20
0.5	1.75	5×10⁸	3/20	2/20	7/20	5/20
Saline	1.75	5×10⁸	13/20	10/20	16/20	12/20
2.0		Nil	0/10	0/10	0/10	0/10
Exp. 2						
1.5	1.5	5×10⁸	3/20	2/20	10/20	5/20
1.0	1.5	5×10⁸	7/20	2/20	13/20	6/20
0.5	1.5	5×10⁸	9/20	10/20	13/20	9/20
Saline	1.5	5×10⁸	13/20	13/20	16/20	18/20
1.5		Nil	0/10	1/10	0/10	1/10
Saline	Tryptic meat broth	5×10⁸	0/5	0/5	0/5	0/5
Saline	Tryptic meat broth	5×10⁸	5/5	3/5	5/5	5/5

* For the sake of brevity these figures are a combination of those of two identical experiments. Other remarks as for table 1.

whether or not α-tocopherol is added to it.

The protection given by peanut oil (with or without α-tocopherol) or by mucin against subsequent infection with mucin and *S. typhi*, is thus a nonspecific effect. Protection of this type, produced by the preliminary injection of a wide variety of substances, is a well known phenomenon, described first by Issaeff (1894) for *Vibrio cholerae* and by Durham (1897) for the typhoid bacillus. The effect is thought to result from the mobilization of phagocytic cells to the site of infection. This was directly demonstrated by Freedlander and Toomey (1928) for subcutaneous infections, following the local application of broth compresses. That such oddly assorted materials as broth, urine, mucin and peanut oil have all been shown to protect against peritoneal infection indicates clearly the nonspecific nature of the effect.

The protective effect of preliminary injections of mucin given subcutaneously is more difficult to explain. The injections are given under the skin of the loin and lateral abdominal wall. The inflammatory reaction produced in this site might easily affect the parietal peritoneum, producing a local leucocytosis, but the possibility of a general blood leucocytosis must also be considered.

Concerning future research on the mode of action of mucin, we feel that full cognizance should be taken of recent work on the chemical fractionation of mucin. This has shown that the action of mucin is not due to a single factor but to a synergic combination of a viscous medium, a particulate residue, and a third factor of three components: a highly active heparin, a less active chondroitinsulfuric acid, and blood group mucoid (Smith, 1950a and b, 1951; Smith et al, 1951; Smith, Gallop and Stanley, 1952; Smith, Gallop, Harris-Smith and Stanley, 1952; Smith and Gallop, 1953; Smith et al, 1953). The over-all activity of mucin is almost certainly due to a combination of different biological properties, and this complex problem should be approached by an investigation of the properties of the individual factors before an attempt is made to use a combination of them. In other words, it would be better to stop using crude mucin, which most workers have done up to the present, and to work at first with components. A start has been made in this direction by investigating certain biological properties of heparin (Lambert and Richley, 1952).

SUMMARY

1. A preliminary subcutaneous or intraperitoneal injection of mucin protects against a subsequent infection with *Salmonella typhi* in mucin.

2. The effect of a preliminary intraperitoneal injection of α-tocopherol in peanut oil on a subsequent infection with *S. typhi* in mucin is not greater than the effect of peanut oil alone.

3. The repercussion of recent chemical studies on the approach to research into the mode of action of mucin has been discussed.

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THE INFLUENCE OF STRAIN, SEX AND AGE OF MICE ON INFECTION WITH *PLASMODIUM BERGHEI*

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While studying the effect of a *Plasmodium berghei* infection on the course of leukemia[1] in mice it was noted that DBA mice and F_1 hybrid mice (C57 Black ♀ × DBA ♂) differed in their resistance to malaria. Of the many investigators who have studied *P. berghei* in mice, none of whom we are aware has used inbred strains. By the use of such homogeneous strains of mice, differences in the reaction to the infection might become apparent and might lead to profitable investigations concerning genetic and nongenetic influences in infection. The present report deals with the course of *P. berghei* infections in inbred DBA/2 and C57 Black mice and their F_1 hybrids, BD F_1 mice.

MATERIALS AND METHODS

The Kasapa strain of *P. berghei* used in these studies was furnished through the kindness of Dr. I. H. Vincke, Elizabethville, Belgian Congo. This strain has been maintained in white mice by serial transfer of infected blood, as previously described.[2]

We are indebted to Dr. Lloyd Law of the National Cancer Institute, National Institutes of Health, for supplying most of the DBA/2 and C57 Black and all of the BD F_1 mice.

One hundred and eighty-four mice were used in these experiments, distributed as shown in table 1.

The malaria donors for all experiments were the so-called "white Swiss" mice. Thirty-five percent to 85% of red cells were infected when the blood was drawn. The donors were bled by heart puncture; heparin was used as the anticoagulant. Whole blood was diluted 1:100 with physiological saline (0.85%), and each mouse received 0.1 ml of the diluted inoculum through a tail vein. All the experimental animals were proven to be infected by at least one Giemsa-stained blood smear in which parasites were positively identified. In several groups of animals the course of the infection was followed regularly by blood smears. All the animals receiving the infection died before the 36th day, except 2 mice (BD F_1) which were killed on the 46th day.

RESULTS

The survival data for all 184 mice used in all the series have been summarized in figure 1 and table 2. BD F_1 mice of various ages and both sexes lived significantly longer than either parent strain, and the C57 Black mice lived significantly longer than the DBA strain animals. There was considerable spread in the range of survival days. However, the significant differences in survival rates are readily apparent when judged from the day on which half the animals of each strain were dead. Especially noteworthy is the fact that *100%* of the DBA mice and *97%* of the C57 Black mice were dead *before* half the BD F_1 mice died.

There are two variables in these experiments that are apparent in addition to the genetic background of the hosts. These are sex and age. The data in table 2 show that the differences in survival among the males of the two inbred strains and their F_1 hybrid were significantly different ($P < 0.01$).

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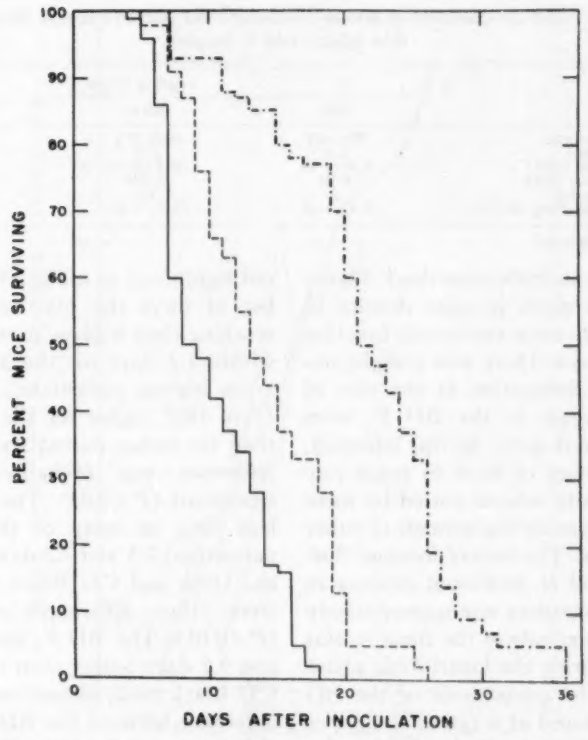

FIG. 1.—Percent of mice surviving as a function of time after inoculation with *P. berghei* in mice of the DBA and C57 Black strains and their BD F_1 hybrid.
—— DBA; - - - C57 Black; -·-·- BD F_1

However, male C57 Black mice survived a mean of 3.4 days less than the females (16.6 ± 0.8 days) of this strain, a difference which is significant ($P < 0.01$). There were too few female DBA or BD F_1 mice for valid comparisons of the effect of sex on survival.

TABLE 1.—*Age, sex and strain of mice used in experiments.*

	Strain of mice		
	DBA	BD F_1	C57 Black
Number of animals:			
Male	70	46	25
Female	6	17	20
Total	76	63	45
Age	1–4 mo.*	2–5 mo.	2½–7 mo.
Number of groups	9	9	6
Number of animals in each group	5–18	3–15	4–10

* One group 12 months old.

In individual series where mice of of the same strain and sex, but of different ages, were inoculated at the same time, there was an increase in survival with increased age (table 3). The mean increase in survival per month increase in age was less than one day. The effect of age differences among all the mice would scarcely influence the over-all mean values in table 2.

Six DBA, 7 BD F_1 and 7 C57 Black mice, all females and 4 months of age were inoculated with parasitized blood on the same day from the same donor (the latter's parasitemia was 35%). Blood smears were made and examined daily, with two lapses, beginning the day after inoculation and continuing

TABLE 2.—*Summary of data on survival of DBA, BD F_1 and C57 Black mice infected with* P. berghei.

	Strain of mouse		
	DBA	BD F_1	C57 Black
Number of animals	70 ♂, 6 ♀	46 ♂, 17 ♀	25 ♂, 20 ♀
Mean age (mo.)	2.9	3.6	3.7
Mean survival (days)	9.9±0.46	20.5±0.87	14.8±0.76
Range of death (days)	4–19	7–36*	6–27
50% dead by day	8	21	13
Mean survival (days, males)	9.9±0.44	19.7±1.14	13.2±0.71

* 2 killed after 46 days.

until all the animals were dead. Figure 2 shows the mean parasite density in animals of the same strain as a function of time in days. There was a slight unaccountable fluctuation in the rate of parasite increase in the BD F_1 mice which occurred early in the infection, but the number of days to reach any parasite density seldom varied by more than 2 days among the animals of either of the strains. The rate of increase during the period of maximum increase in numbers of parasites was approximately the same in animals of the three strains (table 4). During the logarithmic phase of increase the parasitemia of the BD F_1 mice increased at a rate of 1.2% per day less than that of the DBA mice and 1.5% per day less than that of the C57 Black mice; these differences are not significant at a 5% level. The number of days the mice survived after reaching their highest parsitemia varied within 1.2 days for the 3 strains. The mean highest parasitemia reached was 17 to 18% higher for the BD F_1 mice than for either parent strain, and this difference was probably statistically significant ($P<0.05$). The BD F_1 mice had 50% or more of their red cells parasitized 7.5 and 7.2 days longer than the DBA and C57 Black mice, respectively; these differences are significant ($P<0.01$). The BD F_1 mice lived 10.1 and 9.2 days longer than the DBA and C57 Black mice, respectively. The chief difference between the BD F_1 mice and either parent, then, was that they were able to tolerate a higher parasitemia for a longer period of time.

TABLE 3.—*The influence of age on survival of male mice infected with* P. berghei.

Strain of mice	Age (mo.)	Number	Mean survival time (days)
DBA	2	7	6.6
	2½	6	7.7
	12	6	13.2
C57 Black	2½	10	10.6
	3	9	13.1
	7	6	18.0

DISCUSSION

There have been numerous studies of the course of *P. berghei* infections in non-inbred laboratory mice.[3–10] In spite of the variety of routes of inoculation, dilution of the inoculum, age, and sex of

3. Vincke, I. H. and Van Den Bulcke, M. A. 1949, Observations sur l'entretien de la souche

TABLE 4.—*Summary of data on the course of* P. berghei *infections in 4-month-old female DBA, C57 Black, and BD F_1 mice.*

	Strain of mouse		
	DBA	BD F_1	C57 Black
	6	7	7
Mean day of 1st detectable parasitemia	1.0	1.0	1.4
Per cent increase parasitemia/day	6.3±0.9	5.1±0.3	6.6±0.7
Mean highest parasite count (%)	64±5	82±6	63±5
Mean days from highest parasitemia to death	2.5±1.1	1.6±0.6	1.3±0.2
Mean days with parasitemia >50%	2.8±1.1	10.3±0.9	3.1±1.1
Mean survival (days)	13.8±1.24	23.6±1.1	14.7±1.9

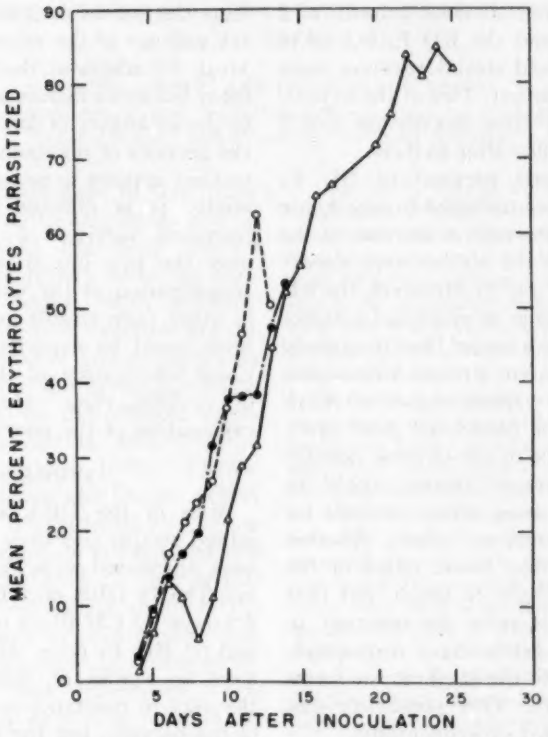

FIG. 2.—Mean daily parasitemia after inoculation with *P. berghei* in mice of the DBA and C57 Black strains and their BD F_1 hybrid.
○—DBA; Δ—BD F_1; ●—C57 Black.

the host used by these investigators, there is general agreement that laboratory mice survive 6 to 10 days after the appearance of parasites in the peripheral blood. The DBA mouse in our experience behaves as the common laboratory mouse. The C57 Black

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4. Baldi, A. 1950, Sulla quadro anemico nell'infezione da "*P. berghei*" (Vincke e Lips). Riv. di malariol. **29**: 349–356.
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6. Ramakrishnan, S. P. and Prakash, S. 1950, Studies on *Plasmodium berghei* n. sp. Vincke and Lips, 1948. I. Variations in susceptibility in albino mice. Indian J. Malariol. **4**: 361–367.
7. Ramakrishnan, S. P. and Prakash, S. 1950, Studies on *Plasmodium berghei* n. sp. Vincke and Lips, 1948. II. Morphology, periodicity and pathogenicity in blood induced infections in mice, rats and garden squirrels. Indian J. Malariol. **4**: 369–375.
8. Schneider, J. and Montezin, G. 1950, Technique d'utilisation au laboratoire de la nouvelle souche de *Pl. berghei* pour l'étude et la recherche des médicaments antipaludiques. Bull. Soc. path. exot. **43**: 144–148.
9. Schneider, J. and Schneider, C. R. 1950, *Pl. berghei:* absence "d'incubation' au cours du passage de souris à souris par voie intraperitoneale. Bull. Soc. path. exot. **43**: 324–332.
10. Findlay, G. M. and Howard, E. M. 1952, Cortisone and *Plasmodium berghei* infection in mice. Nature, **29**: 547.

mouse, however, survives a mean of 5 days longer, and the BD F_1 hybrid of these two inbred strains survives more than 10 days longer. Two of the hybrids lived 36 days after inoculation and 2 others were killed after 46 days.

The increased survival of BD F_1 mice cannot be attributed to any innate resistance to the rate of increase of the parasite, since the strains were similar in this respect (fig. 1). However, the BD F_1 mice were able to withstand a higher parasitemia for a longer time than could either of the parent strains. A resistance to one of the various causes of death from malaria is considered more probable. Resistance to one of these possible causes, e.g. anoxic anemia, could be examined by using other methods for producing anemia or anoxia. Another possibility is that tissue forms of the parasite contribute to death and that some strains of mice are resistant to these without exhibiting a corresponding resistance to the erythrocytic forms of the parasite. This possibility can also be examined experimentally.

From examination of the variability in survival, it is obvious that there are other causes of variability in survival of no less significance than the genetic constitution of the host. It is apparent from the limited data in this paper that sex and age of the mice influenced survival. In addition, there are probably other unknown factors which contribute to the variability of survival. A study of the genetics of resistance to death from malaria appears to be worthy of further study. It is difficult to explain the increased survival of the F_1 hybrid over the two inbred strains of mice. Investigation of the course of malaria in other pure strains and their F_1 hybrids could be expected to give additional information of the applicability of "hybrid vigor" (heterosis) as an explanation of the present observation.

SUMMARY

Mice of the DBA and C57 Black inbred strains and their BD F_1 hybrid were inoculated with *Plasmodium berghei*. The 76 DBA mice lived a mean of 9.9 days; 45 C57 Black mice, 15.9 days; and 63 BD F_1 mice, 20.5 days. There were no significant differences among the mice in resistance to multiplication of the parasite, but the F_1 hybrid mice were able to survive longer with higher parasitemia than either of its parents. Female mice of the same strain survived longer than males, and survival increased with the age of the mice.

STUDIES ON THE RESPIRATORY METABOLISM OF *BALANTIDIUM COLI*

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While the metabolism of free-living ciliates, of parasitic flagellates, and of malarial parasites has been studied rather extensively, very little has been done in this respect with parasitic ciliates. The only data available are the respiratory studies of Daniel (1931) on *Balantidium suis*, the investigations of Hungate (1943) on the cellulose digestion of rumen ciliates, and those of Masson and Oxford (1951), Oxford (1952), and Sugden and Oxford (1952) on the growth requirements and carbohydrate metabolism of holotrich ciliates parasitizing ruminants. In view of this paucity of information a study of the respiratory characteristics of *Bal. coli*, probably the only parasitic ciliate occurring in man, was undertaken. It is evident that a collection of data on various protozoa is significant from a comparative biochemical standpoint, and furthermore, it can be hoped that studies on pathogenic forms may yield information applicable at some future date to a rational approach to chemotherapy.

MATERIALS AND METHODS

The derivation and some characteristics of the strain of *Bal. coli* used in the present study have been described previously (Neghme, Agosin, Christen and Rubio, 1952). The parasites were cultivated in 150 x 20 mm test tubes at pH 7.2 in the liquid medium described by Rees (1927); it was enriched with rice starch instead of rice powder. The ciliates were accompanied by a bacterial flora consisting essentially of *Escherichia coli* and *Proteus vulgaris*. Bacterial growth was to some extent reduced by adding 50 I.U. of streptomycin sulphate and 50 I.U. of crystalline penicillin G sodium (Merck) to each ml of medium before each transfer. The antibiotics were dissolved in the same Ringer's solution which was used in the preparation of the medium (NaCl, 6.5 g; KCl, 0.14 g; $CaCl_2$, 0.12 g; $NaHCO_3$, 0.20 g; Na_2HPO_4, 0.01 g; and sufficient distilled water to make 1000 ml).

The ciliates, immediately prior to their use for respiratory studies, were partially separated from the bacteria by the following procedure. Two ml each of the bottom material from 6 to 8 tubes were pooled and centrifuged for 5 minutes at 10 to 15 G. The supernatant was discarded and an equivalent volume of sterile medium was used to resuspend the sediment, which was then recentrifuged. This operation was repeated 5 to 6 times. After the final centrifugation the volume was adjusted to 3 ml. The material was thoroughly mixed, and the ciliates counted according to a previously described technique (Neghme et al 1952). Each experiment was accompanied by a control experiment for the respiratory activity of the residual bacteria. In order to obtain the latter, material from tubes prepared in exactly the same way as the *Bal. coli* tubes was used. These tubes had been inoculated at the time of transfer not from the *Bal. coli*-containing bottom material of the stock tubes, but from the *Bal. coli* free supernatant. Both types of experimental tubes were incubated for an equal period, usually 24 to 48 hours, before being concentrated as described above. In a few cases, however, the rate of oxygen consumption was determined on material harvested from 96-hour cultures. During the actual respiration experiments bacterial activity was further held in check by adding both to the *Bal. coli* and the bacterial control vessels an equal amount of streptomycin (25 to 50 I.U. of streptomycin sulphate [Merck] dissolved in 0.05 to 0.01 ml of Ringer's solution).

The respiration experiments were carried out according to the conventional Warburg technique. The flasks used had a capacity of approximately 6 ml, and the final fluid volume was always 1 ml. Between 7,200 and 15,300 ciliates were introduced into each flask. The determinations of the oxygen consumption were done at 37 and 28 C, with air as gas phase. The respiratory

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TABLE 1.—*Respiratory characteristics of* Bal. coli.

Temperature (C)	Oxygen uptake (mm^3/hr/1000 cells)	R.Q.	Anaerobic respiratory CO_2 (mm^3/hr/1000 cells)		Anaerobic inorganic CO_2 (mm^3/hr/1000 cells)
			Bicarbonate present	No bicarbonate	
37	9.40±0.67 (38)				
28	4.23±0.42 (13)				
37		1.03±0.03 (10)	6.27±0.49 (22)	5.95±1.5 (9)	0.92±0.36 (22)

The values are corrected for bacterial activity and represent average values with the standard error of the mean; the figures in parentheses indicate the number of determinations.

quotient (R.Q.) and the anaerobic CO_2 production were measured only at 37 C. Some of the latter measurements were done with the practically bicarbonate-free culture fluid serving as medium (gas phase: 99.9% Linde nitrogen further purified by passing over heated copper), but in the majority of the anaerobic determinations the bicarbonate concentration of the medium was raised to a final concentration of 0.0033 *M* and 95% N_2 and 5% CO_2 were used as gas phase. Both in the case of the R.Q. and the anaerobic CO_2 determinations the initial and final bicarbonate content of the medium was determined in the usual manner. All experiments were limited to 60 to 90 minutes duration, and readings were taken at 10- to 15-minute intervals.

The inhibitor studies were always accompanied by both an uninhibited *Bal. coli* and bacterial control vessel. The inhibitors were always added to the main compartment of the experimental flasks and again both a ciliate-containing and ciliate-free bacterial control flask were used in each instance. Most inhibitors were dissolved in Ringer's solution in such a concentration that the addition of 0.1 ml gave the intended final concentration, but 2 methyl-1,4-naphthoquinone was dissolved in a Ringer's solution containing 40% ethanol, resulting in a final alcohol concentration of 4%. Control experiments showed that this alcohol concentration was entirely harmless to the ciliates. When potassium cyanide was used as inhibitor, the CO_2 produced was absorbed with the KOH/KCN mixture of Robbie (1948).

All values obtained were corrected for the respiratory activities of the bacteria derived from the controls described above, and the resulting data were calculated in terms of mm^3 gas/hour /1000 ciliates.

RESULTS

The normal respiratory characteristics of *Bal. coli* are summarized in table 1. It is evident that the ciliates are capable of consuming considerable amounts of oxygen despite the fact that they live normally in surroundings where little, if any, oxygen is present. As figure 1 shows, the rate of oxygen consumption was uniform throughout the entire experimental period, indicating that oxygen of atmospheric tension did not harm the parasites. This is also indicated by the fact that motility of the ciliates was quite normal at the end of the experiments, as far as can be judged from microscopic inspection. These observations are in accord then with those of Schumaker (1931), who found *Bal. coli* remarkably resistant to even abnormally high oxygen tensions. Temperature, as for any poikylothermic animal, had a profound influence on the rate of oxygen consumption (table 1). The Q_{10} for the temperature range 37 to 28 C was 2.6, a figure within the usually encountered range.

The age of the cultures had, within

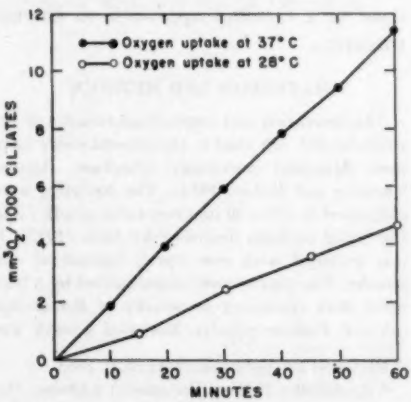

FIG. 1.—Oxygen consumption of *Bal. coli*. Each point represents the average of 10 determinations.

the range studied, little influence on the rate of oxygen consumption. Material from 24- and 48-hour cultures yielded the same average figures (9.9 ± 1.7 and 10.8 ± 0.6 mm,^{3} respectively), but in three experiments with 96-hour cultures a slightly lower value, 7.8 ± 0.1 mm,^{3} was found.

The R.Q. of *Bal. coli* was very close to 1.0. It may nevertheless be significant that the average figure was slightly above 1.0. A similar situation has been found in many parasites and may possibly indicate that some aerobic fermentations are present.

The data discussed have all been corrected for bacterial activity. It may be noted that this correction was relatively small, the gaseous exchanges of the bacterial controls lying in almost all cases between 10 and 20% of those of the ciliates. The R.Q. of the bacteria was 0.83 ± 0.07; that is significantly different from that found for the ciliates.

The anaerobic survival of the ciliates was excellent both in the presence and absence of bicarbonate. Their motility was unimpaired after a 90-minute period. This, of course, is not surprising since the anaerobic faculties of this parasite have been well established in culture studies (for example, Schumaker, 1931; Tanabe and Komada, 1932, and others). In the absence of oxygen, the ciliates produced relatively large amounts of true respiratory carbon dioxide both in the presence and absence of bicarbonate, indicating definitely that their anaerobic metabolism does not consist simply of a degradation of carbohydrates to lactic acid. The acid production, as indicated by the liberation of carbon dioxide from bicarbonate, was moderate, but the nature of the acid or acids produced has not been established.

A summary of the effect of various inhibitors on the aerobic and anaerobic gaseous exchanges of both *Bal. coli* and the bacterial flora is shown in table 2. It is quite evident that in cases where the same inhibitors were employed aerobically and anaerobically, the effect was most pronounced in the latter case. An exception is DL-glyceraldehyde, which proved relatively ineffective under both sets of conditions. It is also quite clear that the inhibitory action of at least some of the compounds was entirely different in respect to the ciliates and the bacteria. Some of the inhibitors. iodoacetamide, sodium arse-

TABLE 2.—*Effect of some inhibitors upon the aerobic and anaerobic gaseous exchanges of* Bal. coli *and its associated bacterial flora.*

Inhibitor	Number of experiments		Concentration	Percentage of inhibition	
	Aerobic	Anaerobic		Aerobic	Anaerobic
Iodoacetamide	2		*M*/200	99 ± 1.58 (84 ± 4.3)	
	4		*M*/1000	81 ± 2.7 (76 ± 14.7)	
	3	4	*M*/5000	49 ± 11.0 (57 ± 4.4)	84 ± 11.7 (6 ± 6.0)
Sodium arsenite	4	4	*M*/100	62 ± 1.4 (73 ± 5.3)	76 ± 6.0 (0.0)
2 methyl-1,4-naphthoquinone		4	*M*/1000		69.2 ± 5.8 (37 ± 1.4)
DL-glyceraldehyde	2	4	*M*/100	16 ± 1.2 (48 ± 10.0)	20.5 ± 8.5 (15 ± 12.5)
	2		*M*/200	6 ± 7.5 (27 ± 34.0)	
Sodium malonate	3		*M*/100	42 ± 5.5 (0.0)	
Sodium fluoroacetate	4		*M*/200	46 ± 8.3 (32 ± 8.5)	
Sodium fluoride	4		*M*/10	44 ± 2.1 (35 ± 10.0)	
	2	4	*M*/100	5 ± 6.3 (17 ± 1.2)	55.2 ± 2.4 (64.7 ± 18.2)
Nitrofurazone derivative 185*	4		*M*/200	49 ± 5.7 (97 ± 2.5)	
Potassium cyanide	4		*M*/2200	67 ± 5.6 (79 ± 6.2)	

Average values with the standard error of the mean; the values in parentheses refer to the bacterial flora.
* (5-nitro-2-furaldehyde 4-(3-diethyl-aminopropyl) semicarbazone hydrochloride.

nite, and possibly 2-methyl-1,4-naphthoquinone, are usually considered as sulphydryl inhibitors acting largely on the glycolytic processes. These three compounds were quite effective against the respiratory processes of the ciliates. Malonate and fluoroacetate, commonly regarded primarily as inhibitors of the tricarboxylic acid cycle, did reduce the oxygen consumption of *Bal.* markedly. Fluoride, of which the point of attack in whole organisms is somewhat uncertain, had to be raised to a very high concentration in order to inhibit the oxygen consumption noticeably; it was, however, somewhat more active anaerobically. Nitrofurazone derivative 185 was fairly effective, but its mode of action is not known at present. Cyanide, a typical inhibitor of heavy metal catalysis, did reduce the oxygen consumption to a marked extent.

In general, there was a close correlation between inhibition and reduction of motility except in the case of DL-glyceraldehyde. Under the influence of the highest concentration of this compound that was used, the motility was greatly reduced, but the respiration was influenced only little. Cyanide reduced motility, and the inhibition was reversible. If, after the termination of the experiments, a drop of the medium containing cyanide was exposed to air on a slide, motility of the ciliates was rapidly renewed, evidently because of the removal of cyanide by evaporation.

DISCUSSION

There can be no question that the greatest advances in our knowledge of the metabolism of ciliates have been achieved with *Tetrahymena* (Kidder and Dewey, 1951; Seaman, 1950 and 1951; Ryley, 1952). This is no coincidence but quite evidently resulted because this organism can be grown in large numbers without bacteria. Some success in purifying free-living ciliates sufficiently from contaminated cultures for metabolic studies has been reported (Humphrey and Humphrey, 1948; van Grembergen and Reynaerts-De Pont, 1952), but the question whether data are valid when gained from impure cultures of parasites grown at high temperatures and therefore heavily contaminated with bacteria deserves critical discussion. It must be realized that the centrifugation of numerous small particles such as protozoa will by necessity entrap an unknown number of bacteria and mechanically carry them into the deposit. It seems hence very dubious that the protozoa can be freed quantitatively from bacteria by even carefully controlled centrifugation procedures, and it seems very probable that centrifugation of protozoa-containing and protozoa-free control tubes would not yield the same number of bacteria in the final deposit. This source of error has been minimized in our experiments, because we centrifuged, in both sets of tubes, the starch-containing bottom material. Since the number of starch granules was far in excess of the ciliates, it appears probable that the mechanical carrying-down of bacteria was similar in both cases. That our corrected data represent essentially the metabolism of the ciliates is further supported by the following observations: (1) The R.Q. of the ciliates and bacteria was different, (2) during the R.Q. determinations, carbon dioxide was released upon final acidification only in the vessels containing ciliates (bicarbonate-free medium), evidently indicating that during the experimental period the ciliates produced an alkaline substance which had bound some CO_2, (3) the reaction of the ciliates and the bacteria to several inhibitors was quite different. While we believe that our data are reasonably accurate, we are quite ready to admit the posssibility that a correction of some of our views may become necessary

once *Bal. coli* is grown in bacteria-free cultures.

The rate of oxygen consumption of *Bal. coli* at 37 C was high. However, the value found at 28 C was of the same order of magnitude as that reported for *Paramecium*, a ciliate of probably approximately the same volume, at comparable or even slightly lower temperatures (older literature in von Brand, 1935, newer data in van Grembergen and Reynaerts-De Pont, 1952). The R.Q. of *Balantidium* was high, slightly above 1.0. Normally, this would be a clear indication for the predominance of carbohydrate metabolism. In the case of parasites such a conclusion is somewhat hazardous unless supporting evidence is available, because most parasites studied hitherto are characterized by the prevalence of aerobic fermentations, and these change the over-all R.Q. in an unpredictable manner. In the present case the presence of a well-pronounced carbohydrate metabolism is probable. The ciliates engulf large numbers of starch grains and store glycogen, as evidenced by the fact that they assume a dark brown color upon addition of iodine solution. We found, furthermore, that they are fairly sensitive to several glycolytic inhibitors, such as iodoacetamide, arsenite, and 2-methyl-1,4-naphthoquinone. The assumption of a pronounced carbohydrate metabolism in *Bal. coli* is in contrast to the assumption made by Daniel (1931) in the case of *Balantidium suis*. He states that despite the starch engulfment the organism relies chiefly on noncarbohydrate materials, because he found an R.Q. of 0.84. It must be pointed out, however, that Daniel (1931) did not carry out any control experiments with the bacterial flora, nor apparently did he take the bound CO_2 into consideration.

The fact that DL-glyceraldehyde had very little influence on the respiration of *Bal. coli* does not contradict the assumption that it utilizes carbohydrates. This compound blocks the conversion of glucose to glucose-6-phosphate by hexokinase (Lardy, Wiebelhaus, and Mann, 1950), a reaction not involved when polysaccharides are utilized. In view of the presence of starch and glycogen in the ciliates and the fact that naphthoquinone was an effective inhibitor, it can be assumed that polysaccharides rather than glucose were used by these organisms. Meyerhof and Randall (1948) have shown that naphthoquinone inhibits mainly phosphohexokinase. This enzyme is involved in the utilization of both polysaccharides and glucose, in the latter case acting below the level of hexokinase. Thus, our experiments do not necessarily indicate the absence of hexokinase. It is entirely possible that polysaccharide-free ciliates would have reacted differently to DL-glyceraldehyde.

The respiration of *Bal. coli* was fairly sensitive to malonate and fluoroacetate, both usually considered as typical inhibitors of the tricarboxylic acid cycle. While we do not consider our data extensive enough to prove the presence of metabolism involving a cycle of the Krebs' type, they do offer a suggestion that some such terminal sequence may occur.

Cyanide was an effective inhibitor, and this is of interest because it indicates that the respiration of *Bal. coli* is dependent on heavy metal catalysis. It is well known that the respiration of free-living ciliates was formerly considered as cyanide-resistant; more recent studies, however, have shown at least a partial cyanide sensitivity (Baker and Baumberger, 1941; van Grembergen and Reynaerts-De Pont, 1952). Insofar as the respiration of parasitic protozoa is concerned, it is quite cyanide-sensitive in leishmanias (von Brand and Johnson, 1947), trypanosomes of the *lewisi* group

(von Brand and Tobie, 1948), and malarial parasites (McKee and Geiman, 1946), but entirely insensitive in trypanosomes of the *evansi* and *brucei* groups (von Brand and Tobie, 1948) and trichomonads (Ninomiya and Suzuoki, 1952).

SUMMARY

1. The aerobic gaseous exchanges of *Balantidium coli* are characterized by a fairly high rate of oxygen consumption and a respiratory quotient of slightly above 1.0.

2. Anaerobically, relatively large amounts of true respiratory carbon dioxide are given off and some carbon dioxide is liberated from bicarbonate by uncharacterized acid.

3. The aerobic respiration is largely cyanide-sensitive.

4. Both aerobic and anaerobic gaseous exchanges are fairly sensitive to glycolysis inhibitors, but relatively insensitive to DL-glyceraldehyde. The anaerobic metabolism is generally more inhibited by lower concentrations of a given inhibitor than the aerobic one.

5. Inhibition produced by malonate and fluoroacetate suggests an aerobic terminal sequence showing some characteristics of the Krebs' cycle.

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IMMUNOLOGIC CHARACTERIZATION OF A MUCINOLYTIC ENZYME OF *VIBRIO CHOLERAE*

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Burnet and Stone[1] demonstrated that fluid expressed from agar upon which *Vibrio cholerae* had been grown was capable of causing desquamation of the mucosa of a guinea pig ileum in vitro. This desquamation was inhibited by antiserums prepared by the injection of the active filtrate into rabbits. Evidence was advanced by the Australian workers which indicated that the mucinase or mucinases (mucinolytic enzymes, filtrate factors) involved were not identical with the receptor-destroying enzymes (RDE) produced by the same organism. Singer, Wei, and Hoa[2] confirmed the previous work as to the sloughing reaction and found that this was inhibited by antiserums produced by immunization with heat-killed vibrios. They concluded that the antibodies produced by the somatic antigens in cholera vaccines protect the guinea pig ileum against the effect of cholera mucinase. Burnet and Stone,[1] on the other hand, found that the mucinolytic factors were not antigenically related to the heat-stable antigens of *V. cholerae*.

The present report deals with the problem of comparative immunogenic characteristics of variously prepared cholera vaccines with regard to the preservation of mucinase in an antigenic state.

MATERIALS AND METHODS

The basic technique employed was that of Burnet.[3] The test substrate for mucinase activity was ovomucin (prepared by the method of Gottshalk and Lind,[4] the end point being determined by whether the test fluids gave a fibrous precipitate on the addition of protamine sulfate. Ovomucin solutions were standardized by dilution in cold physiologic saline to that highest dilution at which a typical precipitate would form upon the addition of 1 drop of 0.1% protamine sulfate to 0.25 ml of solution. The standard test solution of ovomucin was adjusted to twice this strength.

The routine method for obtaining mucinase was that of Burnet and Stone.[5] A stock strain (P93A) of *V. cholerae* was grown for 24 hours at 37 C on the surface of brain-heart-infusion agar (1%), pH 7.5. The fluid from the agar was expressed through cheesecloth, clarified by centrifugation, and filtered through a Seitz pad.

For titrations of mucinase activity, filtrates were diluted in 5% normal rabbit serum (since eventual titrations were to involve serum, and this appears to be an important conditioning factor). From each of the dilutions of serum a volume of 0.25 ml was added to an equal volume of

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the described standard ovomucin solution in Kahn tubes. These mixtures were incubated for 30 minutes at 40 C. At the end of this time the tubes were chilled for a few minutes to inhibit further enzymatic action, and one drop of the protamine solution was added to each tube. If the ovomucin was intact, long threads formed a tight coagulum. With less ovomucin only very short threads of precipitate formed. The titer of mucinase was recorded as the highest dilution of the filtrate which destroyed the ovomucin to the extent that no thread-like precipitate formed.

EXPERIMENTAL

Quantitative interrelationships between enzyme and enzyme-inhibiting antibody.—Estimation of the antimucinase content of antiserums is largely dependent upon the question of quantitative interaction between the enzyme and the anti-enzyme. In order to establish a baseline for comparisons, an experiment was devised in which varying concentrations of mucinase were allowed to interact with different amounts of antimucinase.

Rabbits were injected intravenously according to a customary time pattern, with increasing amounts of active mucinase-containing filtrates. On the tenth day after the last injection the rabbits were bled and the serums pooled for testing.

A two-step procedure was employed for the demonstration of neutralization, this procedure having been established by a long series of exploratory experiments to determine the most suitable conditions for this demonstration. In the first step (immunologic) rabbit antimucinase serum diluted in 5% normal rabbit serum was mixed with suitable dilutions of mucinase-containing filtrate, the mixtures being held at 4 C for 15 hours. In the second step (enzymatic) the standard ovomucin was added for a second incubation at 40 C (2 hours). The residual mucinase content from the immunologic phase was recorded as the highest dilution of the filtrate factor which destroyed the standard amount of ovomucin. The units of mucinase neutralized by antiserums were calculated from the results obtained with normal rabbit serum, as opposed to rabbit immune serum under precisely the same conditions. Thus, if the mucinase titer in the presence of normal rabbit serum were 1:800 and that with a given quantity of antimucinase rabbit serum were 1:200, it would be considered that 4 units of mucinase had been neutralized by the amount of immune serum employed. For practical purposes four-fold differences may be held to be significant.

The results of a quantitative experiment are shown in table 1. Within the obvious limitations of this type of test, and over the confined range involved, it would appear that the results of interaction of anti-enzyme and enzyme were roughly quantitative, irrespective of the

TABLE 1.—*Neutralization of mucinase by specific antiserum.*

Dilutions of antiserum	Mucinase units								Indicated neutralization	
	1	2	3	8	16	32	64	128	Units	Units/ml serum
1:10	++	++	++	++	++	++	++	+	128	1280
1:20	++	++	++	++	++	++	+	−	64	1280
1:40	++	++	++	++	++	+	−	−	32	1280
1:80	++	++	++	++	+	−	−	−	16	1280
1:160	++	++	+	−	−	−	−	−	4	640
1:320	++	++	+	−	−	−	−	−	4	1280
1:640	+	−	−	−	−	−	−	−	1	640
Control	−	−	−	−	−	−	−	−	—	—

Antiserum: Pooled serums from 2 rabbits immunized with mucinase-containing filtrate.
Reaction: Antiserum diluted in 5% normal rabbit serum, various dilutions being mixed with various quantities of filtrate factor (the potency of which had been established previously). After 15 hours at 4 C, standard amounts of ovomucin were added for a further incubation, 2 hours at 40 C.
Results were recorded in terms of typical reaction upon the addition of protamine sulfate, that is, a positive result describes the fact that mucin remained unaffected by the enzyme, presumably because the enzyme had combined with antibody and was thus rendered inactive.

TABLE 2.—*Antimucinase values of serums of variously immunized rabbits.*

Immunizing antigens	Agglutinin titers	Mucinase titer in serum-mucinase mixtures	Units of antimucinase/ 0.0125 ml serum
None (normal rabbit serum)	0	1600	0
Filtrate factor (freshly prepared)	1:640	25	64
Filtrate factor, lyophilized	1:640	25	64
Filtrate factor, heated 56 C, 30 min.	1:640	1600	0
Vibrios from agar culture, washed	1:640	200	8
Vibrios from agar culture, washed, lyophilized	1:640	200	8
Vibrios from agar culture, washed, heated 100 C, 60 min.	1:640	1600	0
Vibrios from agar culture, washed, formalin to 0.3%	1:640	1600	0
Vibrios from agar culture, washed, phenol to 0.5%	1:320	800	2

Serums: In each instance the serums of 2 rabbits immunized with the same antigen were pooled.
Titers: The mucinase titers of serum-mucinase mixtures are with reference to the result obtained with normal rabbit serum, this being regarded as lacking mucinase-inhibitor. Various rabbit serums were employed at a dilution of 1:20. Agglutinin titers were determined against a suspension of washed, viable *V. cholerae*.

concentrations involved.

Development of mucinase-inhibiting antibodies following immunization of rabbits with various preparations.—Various immunizing preparations (vaccines and mucinase-containing solutions) were used in the immunization of rabbits to determine the amounts of antimucinase which might be developed. Although the vaccines were strictly comparable as to vibrio content, the amounts of filtrate factor must be regarded as independent, since there is no method for adjusting antigenic levels as between these dissimilar preparations. Hence, the results in such comparisons are largely qualitative in character.

The results of a simultaneous series of immunizations are shown in table 2. Two rabbits were used for each of the indicated preparations, their serums being pooled for testing. Immunization with mucinase-containing filtrates gave rise not only to a relatively high titer of antimucinase but to agglutinins as well. The antigenic qualities of the mucinase were not impaired by lyophilization. Inactivated filtrate factor did not stimulate antimucinase production but did give rise to agglutinins. It is evident that the filtrate contains some stable antigen of the vibrio, perhaps as a result of autolysis.

Immunization with washed, viable vibrios stimulated the production of agglutinins but the amount of antimucinase was quite low. Similar preparations treated with heat, phenol, or formalin also gave agglutinins but little or no antimucinase. There was no correlation between agglutinin titer and antimucinase titer.

Mucinase content of various experimental cholera vaccines.—The foregoing results can be interpreted as indicating that high antigenicity can be expected with mucinase only when it is in a native state. Thus, problems of production of a lifeless vaccine containing mucinase are concerned with the preservation of the filtrate factor in active form.

The stability of mucinase in liquid form was determined under a variety of conditions. In a series with varied temperature-time combinations a loss of at least 75% of the activity was obtained at each of the following points.

45 C, 2 hours
37 C, 4 hours
21 C, 48 hours
4 C, 2 weeks
−20 C, 50% loss in 8 weeks

Similar losses within 30 minutes were observed upon the addition of formalin to 0.3% and of phenol to 0.5%. With merthiolate added to a final concentration of 0.01% the losses were no greater

than those recorded for preparations without preservative.

Lyophilization gave preparations which appeared to be quite stable during an 8-week period of testing. The lyophilized material in sealed ampoules withstood the stress of 100 C for 1 hour without loss of activity upon rehydration.

For the preparation of a stabile vaccine there are then available merthiolate as a bacteriostatic agent, and lyophilization as the stabilizing method. Although the experimental work showed that immunization with the filtrate factor gave rise to good agglutinin titers, it seemed desirable for practical purposes to retain the features of a vaccine containing whole but lifeless bacteria and to add to this the filtrate factor. As a type vaccine, a suspension of washed *V. cholerae* containing 8×10^9 viable bacteria per ml was mixed with an equal volume of filtrate factor. Merthiolate was added to the mixture to a final concentration of 0.01%. This final mixture was then lyophilized, the ampoules being finally filled with dry nitrogen and glass-sealed.

The mucinase titer of the original bacterial suspension was 1:4, that of the filtrate factor 1:1600, and that of the final product 1:1600. Rehydrated lyophilized material gave the same titer. Sterility tests gave no growth. The lyophilized product was subjected to various stresses, such as 100 C for 1 hour. There was no detectable loss of mucinase activity. It may be inferred that activity would be well retained upon long storage at lower temperatures. The experimental vaccine gave good antibody production in rabbits and appeared to be nontoxic for these animals. The vaccine was nontoxic for mice by intracerebral, intra-abdominal, and intramuscular routes.

DISCUSSION

This report has dealt with immunologic matters relevant to the inclusion of mucinase in cholera vaccine. Although Burnet's suggestion that mucinase may participate in the pathogenesis of cholera is most attractive, there is no evidence at this time that such is the case. Furthermore, a number of proteolytic ferments are produced by *V. cholerae*. If enzymes have a role in the pathogenesis of cholera, it is likely that several are involved. The present report has dealt with only one of possibly several substances which might be designated as mucinases.

The present work has substantiated Burnet's observation that the usual cholera vaccine does not upon injection give rise to significant amounts of antimucinase. Hence, the emphasis of these experiments has been directed toward the practicability of the inclusion of a specific labile bacterial product, not highly antigenic save in native form, in an immunizing preparation.

SUMMARY

The studies here reported show that the filtrate factor of *Vibrio cholerae*, a good antigenic agent for the production of antimucinase, can be incorporated in stabile form in a cholera vaccine. Evidence presented here showed that the enzyme responsible for the dissolution of ovomucin is dissimilar antigenically to the heat-stable agglutinogens of the vibrio. Methods for the evaluation of antimucinase were presented.

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